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The Mucoid Phases of the Genus Brucella

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IT IS NOT GENERALLY known or appreciated that the species of Brucella dissociate as easily and show a dissociation pattern as complex as that of any other microörganisms. Henry[1] has described five dissociated colony phases obtained from *Brucella abortus*.. One of these possessed mucoid characteristics. He designated this phase with the letters RB (rough brown), on account of the chestnut brown appearance of isolated colonies. They were smaller than R (rough) phase colonies and occasionally mucoid in consistency. Henry expressed the opinion that the pH of the medium was the factor which influenced the development of mucoid colonies.

Previous to Henry's description of the RB colony phase, Plastridge and McAlpine[2] had noted a mucoid growth of the three species of Brucella in a liquid medium after a ten-day growth period. Nearly all of their data pertaining to the mucoid growth are based on its appearance in liquid mediums and its biochemical and antigenic characteristics. Apparently, no attempt was made to separate the mucoid phase from the many other phases that might possibly be present in broth cultures or to study its colonial characteristics. They observed that the appearance of the growth on liver-infusion agar slants was indistinguishable from the S (smooth) phase.

Stearns and Roepke[3] demonstrated that M (mucoid) phases of *Br. abortus* had a higher electrophoretic mobility than the S phase and were agglutinated by antiserum prepared from the S phase. Their cultures appeared to be pathogenic for guinea pigs.

The different mucoid or nearly waxlike dissociated phases which occur in strains of each species have passed unobserved or, if seen, given little attention. They are, without a doubt, the most interesting and, from an immunogenic viewpoint, the most important of all the growth phases of this genus.

There are two possible explanations for the absence of informative knowledge pertaining to the mucoid and waxlike phases of Brucella. One is the failure to use the proper lighting system without which it is difficult to distinguish this phase from others or differentiate one mucoid phase from another within the species. The other is that, if casually observed under a low-power microscope, they might easily be mistaken for the R phase. Since this phase of most bacteria is considered unimportant from an antigenic or immunogenic viewpoint, it may have been discarded when encountered.

Before giving a description of the mucoid phases of Brucella, it is essential that a satisfactory procedure for their identification be described, and for the purpose of comparison, that a brief description be given of the colonial and other characteristics of the best known and most often seen

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phases of Brucella, namely, the S and R phases.

The only known procedure by which the S and many dissociated phases, with possibly one exception, can be distinguished one from the other is from the examination of well-separated, four-day-old colonies incubated at 37 C. on a clear medium such as tryptose agar in Petri plates. The colonies must be observed and manipulated under a low power (12.5) stereoscopic microscope. The light source and manner of illuminating the colonies for visual examination is very important. A small microscope lamp possessing a focusing lens is the best light source. The lamp is placed 18 inches in front of the microscope and pointed downward at an angle of approximately 45 degrees. The light rays from the filament are focused on a flat surface mirror placed in front of the lamp. The rays are reflected, at an oblique angle, up under the glass-plate stage of the microscope, into the solid culture medium. When a Petri plate containing solid culture medium is placed on the stage, the light rays strike the bacterial colonies on the surface of the medium at different angles. Unlikeness in colonial appearance can easily be detected from light absorption differences.

Colonies of the S phase of *Br. abortus* on tryptose agar plates, when widely separated from each other, are from 1.0 to 2.0 mm. in diameter on the fourth day of incubation at 37 C. They are circular, smooth, convex, entire, glistening, translucent, and of soft consistency. The color varies from a light bluish-green to a cloudy green; the centers may become slightly opaque with age. S colonies of *Brucella suis* resemble those of *Br. abortus*, but are slightly larger in diameter and are more cloudy green in color. The entire colony becomes slightly opaque with age, losing most of its original green color. S colonies of *Brucella melitensis* meet the description set forth for *Br. abortus* with the exception of size. Isolated colonies seldom reach more than 1.5 mm. in diameter.

The S phase of all species suspends readily in 0.85 per cent NaCl solution and makes fairly stable suspensions.

The extreme dissociated phase of Brucella is perhaps the R phase. This phase, when seen as large separate colonies, is easily recognized regardless of the species. The colonies are circular, raised, opaque, and have a ground glass appearance when scattered by a wire pointer. In consistency, they are soft but slightly drier than the S phase. The writer has never observed daughter colonies developing in R phase colonies.

The R phase grows as a lacelike pellicle in liquid culture medium. When slightly agitated, the pellicle breaks into small granules and falls to the bottom of the tube. When suspended in 0.85 per cent NaCl solution, R cells rapidly clump together and settle to the bottom of the tube. R phase cultures of all the species are not pathogenic for experimental animals and are almost inactive in the production of serum antibodies.

When cells of the S phase of the three species of Brucella are inoculated into tubes of tryptose broth of pH 6.8 and incubated at 37 C. for ten days or longer, without shaking, the growing cells may undergo considerable change. The changes are recognized best by streaking a small loopful of the broth culture over the surface of tryptose agar plates in such a manner as to obtain colonies well separated from each other. They should be examined after four days of incubation at 37 C., according to the method described herein or by Henry[1].

Examination of the growth on inoculated plates may reveal colonies of the S phase, two or three I (intermediate R) phases, the R phase, and possibly a small number of colonies of one or more mucoid phases.

During incubation in broth, the culture may dissociate only into the R phase according to colony morphology. When this occurs, one should inoculate a fresh tube of broth with the S culture. Often, mucoid colonies appear in only small numbers among the other colonies that arise on seeded agar plates. Occasionally, however, a transfer of a *Br. suis* culture may show a predominance of colonies of one of its mucoid phases.

The different mucoid phases of the species of Brucella that have been isolated and studied by the writer, appear to develop in liquid culture medium (tryptose peptone broth) from the S phase as if by chance. In other words, one cannot predict how soon or how many phases will appear in a single culture.

The mucoid colonies which appear on tryptose agar among other phases are opaque with a slight orange color. They can be easily distinguished from other dissociated phases by their color and consistency.

Thus far, *Br. abortus* has been found to dissociate into three distinctly different mucoid phases. These have been designated as Ma (mucoid *abortus*) phase 1, 2, and 3. Ma 1 colonies might easily be mistaken for S colonies when they are closely packed together on the surface of agar plates. Widely separated colonies vary from 1.0 to 2.5 mm. in diameter. They are circular and slightly raised in the center, with rounded and raised margins. The marginal border of the colony is light bluish-green, but the central area is slightly opaque with a slight orange tint. When a fine wire pointer is swept through the colony and lifted upward, the growth has a tenacious, mucus-like consistency. After more than 100 single colony transfers over a period of one and a half years, this phase has shown no tendency to revert to S or to dissociate into other phases.

Ma phase 1 dissociation can be produced rapidly by growing the S phase in a liquid culture medium to which has been added bovine serum containing growth inhibiting antibodies specific for *Br. abortus*. As soon as the inoculated medium becomes slightly turbid (after forty-eight hours of incubation at 37 C.), a loopful is streaked over the surface of a tryptose agar plate. After four days of incubation at 37 C., most of the colonies which appear on the surface will be found to belong to phase 1.

Colonies of Ma phase 2 are flatter than those of Ma 1. The centers are opaque and have a slight orange color. The outer borders are translucent and without color. The edge of the colony is thin, melting out at the surface of the medium. The consistency of the colony is much like that of Ma 1. Small, closely packed colonies are easily taken for the S phase in that they are gray-green and their consistency is soft. This phase also has shown no tendency to revert to S or to dissociate into other phases. Neither Ma 1 nor 2 develops daughter colonies.

When Ma phase 1 or 2 is grown in tryptose broth, both produce a uniform turbidity throughout the medium. After several days of incubation, the cells settle to the bottom of the tube more rapidly than those of the S phase.

Ma phase 3 colony is circular and convex; its edge is not raised. The size is similar to phases 1 and 2. It is translucent and of faint orange color. The entire colony becomes transparent with age. Well-isolated, large colonies at four days of age at 37 C. are quite tenacious in consistency. They are more waxlike than mucoid. The base of the colony adheres to the surface of the medium. Among the colonies of Ma phase 3 which grow out from succeeding single colony transfers on agar medium will be found a small number of scattered colonies which are light grayish-green and, without further study, might be mistaken for the S phase. These colonies have been picked from among phase 3 growth of many different strains and studied. They have always proved to be a reversion to Ma phase

Fig. 1—Mucoid phase colony of Brucella suis containing daughter colonies. x 12.5.

1. It may be said here that it has not yet been possible to obtain phase 3 growth on either agar or liquid medium completely free from a small number of phase 1 colonies.

Ma phase 3 differs from phases 1 and 2 in another important direction. Small daughter colonies make their appearance on the surface of the mother colony when it is ten days of age. The daughter colonies appear to grow upward from the interior of the colony, bursting through the surface like small buds. As the mother colony becomes more transparent with age, the daughter colonies may be seen below as well as on the surface. Those that grow out near the margin of the mother colony appear to spill over the edge onto the surface of the medium like lava flowing down the side of a volcano. They are more opaque than the mother colony at this age and are slightly greenish. As many as 15 daughter colonies, varying in size from 0.05 to 0.15 mm. in diameter, have been counted in a single mother colony, when twenty-five days of age (four days at 37 C. and twenty-four days at 25 C.). The culture medium on which this phase or the other Ma phases are grown begins to develop a slight brown color when the cultures are four days old. The color is more marked in that part of the medium where the culture is densest. As the culture becomes older, all of the medium in the plate becomes light brown.

Ma phase 3 produces a uniform growth in tryptose broth which on further incubation sediments slowly, forming a ropy mass at the bottom of the tube.

Two mucoid phases have been obtained from the S phase of *Br. suis* during growth in broth. They have been designated as Ms (mucoid *suis*) phase 1 and 2. Widely separated colonies of Ms 1 are 1.5 to 3.0 mm. in diameter, circular, and slightly raised in the center. They have an ivory appearance faintly tinted with orange. The margins of the colonies are slightly raised and rounded. The outer border is more translucent than the center. When a colony is swept with a wire pointer it is mucoid in consistency. It becomes more tenacious with age. As the colony ages at 25 C., it appears more transparent; the change beginning at its periphery and gradually extending inward toward its center. Daughter colonies make their appearance on the sixth day of incubation at 37 C., or on the tenth day at 25 C. after a four-day period of incubation at 37 C. The course of their development is similar in all respects to those that are seen in Ma phase 3, *Br. abortus* colonies. As many as 52 daughter colonies have been counted in a single mother colony when twenty-five days old. An M phase colony containing daughter colonies is illustrated in Figure 1.

Ms phase 1 at first produces a uniformly turbid growth in tryptose broth. After the fourth day of incubation, the suspended cells appear to clump and settle out.

Ms phase 2 colonies are more convex than phase 1. They are opaque with a slight tint of orange. When a colony is swept with a wire pointer, the entire mass clings to the wire and can be lifted from the surface of the medium as though it were soft wax. The entire colony becomes slightly transparent with age and, at the end of thirty days, is no longer opaque. This phase also produces daughter colonies which resemble, in all respects, those seen in Ms 1.

If a portion of the mother colony at thirty days of age is transferred to a plate of tryptose agar, it fails to grow. In other words, the organisms appear to have died out. On the other hand, transfers of the daughter colonies at this time yield vigorous growth.

Ms phase 2 can be induced to revert to the S phase of *Br. suis* by use of the following procedure: A dense suspension of M cells is flooded over a part of the surface of a tryptose agar plate. On examination of the growth after six days of incubation at 37 C. there will be seen protruding from the margins a small number of bluish-green areas of growth. When these are transferred to fresh agar medium, the colonies which appear are the S phase. This has been proved by suspendability in saline solution, agglutinability with specific serum, and pathogenicity.

Ms phase 2 produces a granular growth in tryptose broth which settles rapidly to the bottom of the tube.

Only one mucoid phase of *Br. melitensis* has been seen and studied. It has been designated as Mm phase 1. Colonies of this phase resembled so closely those of the S phase in color, size, and shape, they could not be recognized if one failed to sweep widely spaced ones with a wire pointer. The consistency is that of soft mucus. The content of a colony adheres slightly to the pointer as it is pulled away. Growth of this phase suspends easily in physiological salt solution, but it settles slowly to the bottom of the tube during twenty four hours, forming a compact mass.

Mucoid daughter (Md) colonies which appear on the surface of colonies of M 3 phase of *abortus* and the M phases of *suis*, when streaked over tryptose agar plates, grow like and have the colonial appearance of the S phase. On preliminary examination one would easily mistake either small or large widely separated colonies for S colonies. They have a dull, bluish-green appearance. They are soft and glistening and show no mucoid consistency. Thus far, Md colonies have shown no tendency to revert to the true S or the mucoid phases or to dissociate further on solid or liquid mediums. The M and daughter-colony phase is not unlike those described and studied by Morales-Otero[4]. Cells of the Md colony suspend quite easily in physiological salt solution. The suspended organisms, however, are quite unstable in that they settle to the bottom of the tube as a soft mass during a 24-hour period. The instability of this phase in suspension distinguishes it from the S phase.

All Md phases at first produce a uniformly turbid growth in tryptose broth. After four days of incubation, the cells clump together and slowly settle to the bottom of the tube.

All M phases as well as the Md phase of the species of Brucella remain true to type on differential dye mediums.

The Md colony phase from *Br. abortus* and *Br. suis* fulfills, in many respects, the description which Henry[1] has given to a pseudo-S phase of *Br. abortus*, and identified as S^R. Henry recovered this phase from a cow that had been inoculated with a suspension of an R phase of *Br. abortus*. He stated that this phase was antigenically distinct from the true S and was self-agglutinated and nonvirulent. Its colonial appearance was the same as the S phase.

The writer has not observed Md colonies in the R phase of several different cultures of the species of Brucella when grown on either agar or liquid medium.

All the mucoid phases produce agglutinins in low titer in rabbits for S phase antigen. When the Ma phases are injected intravenously into rabbits in amounts of 1 to 5 mg. of cells, the maximum agglutination titer produced is 1 : 80. The Ms and Md phases produce titers varying from 1 : 640 to 1 : 1,280. Maximum agglutination titer is reached one week after injection.

The M or Md phases apparently are not pathogenic for guinea pigs or cattle. As many as 1×10^{10} live cells have been injected into guinea pigs and 1×10^{12} cells into cattle without producing any evidence of infection. When rabbits are injected intravenously with 1 to 5 mg. of organisms of the M or Md phase, growth-inhibiting serum antibodies for *Br. abortus* are produced; the titers range from 1 : 10,000 to 1 : 80,000.

Suspensions of live cells of certain phases that have been discussed, when injected into guinea pigs, produce a high degree of active immunity against experimental infection from all species of Brucella. Studies which deal with the immunizing potentialities of the mucoid phases of Brucella are now nearing completion and will form the basis of a forthcoming paper.

SUMMARY

It has been found that the species of Brucella dissociate into several distinctly different mucoid or waxlike phases. Three mucoid phases have been obtained from *Brucella abortus*, two from *Brucella suis*, and one from *Brucella melitensis*.

One mucoid phase of *Br. abortus* and those of *Br. suis* give rise to daughter col-

onies which are similar in many respects to S (smooth) phase colonies.

M (mucoid) and Md (mucoid daughter) phase cells, when injected into guinea pigs, rabbits, or cattle, give rise, in their blood serum, to specific growth inhibiting antibodies in high titer and engender in guinea pigs a high degree of active immunity against experimental infection with the species of Brucella.

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The Journal Changes Its Make-Up

With this volume (VII) and issue of the American Journal of Veterinary Research, we call attention to a new section in the make-up, namely, the inclusion of advertising. This addition was approved more than a year ago by the Committee on Journal and the Executive Board, with the understanding that advertising space in this publication would be made available only to a limited and selected group of manufacturers and distributors of scientific apparatus and supplies which are used by research and educational institutions and diagnostic laboratories. We believe that this presentation of newly developed scientific apparatus and supplies will be of real interest to readers of the RESEARCH JOURNAL and of benefit to the advertisers.

Although the number of firms currently participating is small, we are pleased to welcome them, and expect that this section of the RESEARCH JOURNAL will grow in size and interest for everyone.

Beginning also with this volume and issue, the contents are listed on the front cover. We believe this arrangement is more convenient and that it is an improvement over the former method. We hope you will agree with us.

Studies on Brucellosis of Swine
II. Exposure and Reëxposure Experiments with *Brucella Suis*

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BRUCELLOSIS of swine appears to be a somewhat self-limiting disease in so far as the symptoms of abortion and recession of serum agglutination titer are concerned. However, it is necessary to know whether these phenomena result from the production of an active, acquired immunity or whether subsequent reëxposure to the disease will result in active infection. McNutt[1], in discussing immunity in swine to brucellosis, states:

> Not much is known of immunity. Hogs are naturally resistant to both *abortus* and *melitensis* and often show considerable resistance to *suis* strains. Aged hogs are apt to be more severely affected than the very young, and it is thought that recovery from one attack renders the animals less susceptible to subsequent infection. Hence one would suspect that vaccination of young pigs might be of value, but such vaccination has resulted either in failure or inconclusive evidence.

Hadley and Beach[2], basing their opinion on the breeding efficiency of some sows after aborting, concluded:

> Infectious abortion in swine appears to be a self-limiting disease in that a naturally acquired infection usually is followed by immunity.

Results of their studies on immunity produced by vaccination led Hadley and Beach[2] to report that:

> Vaccination is a certain means of conferring active immunity, and gives promise of being an effective means of control, but whether it should be used generally has not been determined.

Apparently, in the past, entirely too much emphasis has been placed on breeding efficiency and lack of abortions in the evaluation of the production of active, acquired immunity to Brucella infection in the various species of animals. From a strictly economic standpoint these factors are definitely important, but from the public health and disease control point of view complete resistance to infection is the most important.

The purpose of this paper is to report the results of reëxposing sows to *Brucella suis* organisms by intravenous, subcutaneous and oral methods. The sows employed in the experiments reported here had been previously exposed, as weanling pigs or adults, to *Br. suis* by various routes.

EXPERIMENTAL METHODS

Group 1 consisted of 19 pregnant sows that had been exposed to *Br. suis* on June 15, 1942, as weanling pigs and were reëxposed, after having completed a normal pregnancy, to a polyvalent inoculum of *Br. suis*. This reëxposure was administered on Aug. 4, 1943, using two routes; *i.e.*, subcutaneous and intravenous. On the basis of their behavior to the primary exposure, these sows were divided into two groups. Group 1(*a*) contained 15 sows which had a previous history of becoming infected while young pigs as evidenced by either the isolation of *Br. suis* from the blood stream or the development of significant serum agglutination reactions or both. Group 1(*b*) contained 4 sows which had a previous history of not becoming infected while young pigs as evidenced by negative findings using the same criteria.

At the time of reëxposure, all the previously infected sows were showing evidence of receding serum agglutination titers or had essentially ceased to react as compared to the titers exhibited after their first exposure. Also, repeated examinations of the blood for *Br. suis* had given negative results for several months.

Group 2 contained 3 second pregnancy sows approximately 18 months old, with a previous history of being free from brucellosis from

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weaning time until exposure on Aug. 4, 1943. These sows were exposed *per os*, subcutaneously, and intravenously with a part of the same inoculum described above.

Group 3 was composed of 12 yearling gilts which were purchased from a herd where there was no previous history of Brucella infection. Nine of these gilts were in various stages of pregnancy, ranging from thirty-five to ninety-five days, and three were open. The 12 gilts were exposed on Aug. 4, 1943, using the same inoculum as was used in the previously mentioned sows, with two different channels of exposure in each gilt; *i.e., per vagina* and *per os*. Thus all of the swine in groups 1, 2, and 3 were exposed on the same day using the same inoculum.

Later 6 yearling gilts, designated as group 4, were purchased from the same source as group 3 and exposed on or near the day they were bred. A polyvalent inoculum made from the transplants of the same cultures as were used to expose groups 1, 2, and 3 was employed. Three routes of exposure were used for each of these six gilts, *i. e., per vagina, per os,* and *per conjunctiva.*

The polyvalent inoculum used in all cases was composed of five cultures which were previously demonstrated to be virulent for guinea pigs and were presumed to be virulent for swine. Two of the cultures were obtained from the Michigan and Minnesota experiment stations. Three of them were isolated at this laboratory, one from the seminal vesicle of an artifically infected boar, one from the epididymis of a naturally infected boar, and one from an aborted fetus from a naturally infected sow. Four of the cultures produced characteristic lesions, agglutination response, and positive bacteriological findings, when injected in small doses in guinea pigs. The fifth culture had been isolated from an aborted fetus on July 31, 1943, only five days prior to its first use as inoculum in these experiments.

The approximate doses of this polyvalent inoculum for the sows and gilts were as follows: intravenous, 3 billion organisms; subcutaneous, 5 billion organisms; *per os*, 5 billion organisms; *per conjunctiva*, 100 million organisms; *per vagina*, 1 billion organisms for group 4, 5 billion organisms for group 3.

Following inoculation, bacteriological examinations of the blood of each experimental animal for *Br. suis* were conducted according to a previously described technique,[3] at intervals of approximately two weeks for two and one-half months and approximately once per month thereafter for four months. Serum agglutination tests were conducted at intervals corresponding to those of the blood cultures. Both the rapid and tube methods of agglutination testing were employed in dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400. Standardized antigens, as supplied by the laboratory of the United States Bureau of Animal Industry at Beltsville, Md., were used for the agglutination tests.

Since intravenous, subcutaneous, oral, vaginal, and conjunctival exposures and reëxposures with pure polyvalent cultures of *Br. suis* yield information on immunity which is of an academic nature, it seemed advisable to repeat the previous type of experiment using a more natural means of inoculation. Consequently, three groups of experimental swine, two of which had been exposed to *Br. suis* previously, were assembled and individually fed small portions of aborted fetuses and fetal membranes from which *Br. suis* had been isolated.

Group 5 consisted of 5 aged sows which had become infected in a natural outbreak of brucellosis. These 5 sows had been kept on our premises for nearly two years following their removal from the infected herd, during which time their agglutination titers had receded to a negative or nearly negative status. The breeding history of these sows had been unsatisfactory during this two-year period. Repeated breeding for farrowing twice a year had resulted in one pregnancy in 2 sows (810 and 827), two pregnancies in 2 sows (832 and 815), and three pregnancies in 1 sow (808). Sow 815 had aborted during the original natural outbreak of disease in the herd.

Group 6 was composed of 6 sows 18 months to 2 years of age which had been artificially exposed to *Br. suis* after reaching sexual maturity (8 to 12 months). Five of these 6 sows (558, 587, 441, 28, 594) developed agglutination titers after the first exposure; 2 sows (558 and 28) gave positive cultures for *Br. suis* in their blood; 1 sow (558) eliminated *Br. suis* in the colostrum at farrowing time. All but 1 of these sows (441) farrowed full term litters during their first pregnancy. At the time of reëxposure, by feeding aborted materials as described previously, 4 of the sows (46, 594, 28, 587) were negative or nearly negative to the agglutination test. Two sows, (441 and 558) were continuing to show a relatively high agglutination response, but *Br. suis* had not been isolated by repeated examinations of the blood of any of these 6 sows for several months.

Group 7 was made up of 5 sows negative to the agglutination test at the time of purchase from a herd of swine known to be free from brucellosis, as measured by all available criteria. These sows were exposed by the same method as those in groups 5 and 6, except that they were fed the aborted materials at or near the day of breeding.

Following such exposures and reëxposures, bacteriological examinations for *Br. suis* of the blood of each experimental animal in all

Table 1—Results of the Subcutaneous or Intravenous Reëxposure of 15 Sows in Group 1 (a)

SOW NO. ROUTE RE-EXP	PRE-RE-EXP TITER	AGGLUTINATION TITERS AND BLOOD CULTURAL RESULTS TIME IN DAYS AFTER RE-EXPOSURE 14	29	41	55	70	101	139	202	FARROWING RESULTS	COLOSTRUM TITER	BR. SUIS CULTURES AT PARTURITION OR AUTOPSY
903 I. V.	++P-- NEG	++PT- NEG	++PT- POS	+++PT NEG	++++P NEG	+++PTT NEG	+P--- NEG	P---- NEG				
947 I.V.	+PT-- NEG	PT--- NEG	T---- NEG	+PT-- NEG	PT--- NEG	+PT-- NEG	PT--- NEG	+P--- NEG	-----	NORMAL	-----	NEGATIVE
913 I. V.	+P---- NEG	+++PT NEG	+++PT NEG	++++P NEG	+++++ POS	+++PT NEG	++PT- NEG	+P--- NEG	+P---	NORMAL	NOT DONE	"
911 I. V.	++PT- NEG	++PT- POS	+++PT POS	++++P POS	++++P NEG	++++P NEG	++P-- NEG	+P--- NEG	+P---	"	++++P	"
915 I. V.	++P-- NEG	++PT- NEG	++PT- NEG	+++PT NEG	++++P NEG	++++- NEG	+T--- NEG	----- NEG	+PT--	"	+++++	"
917 I. V.	+---- NEG	+++PT NEG	+++PT POS	+++PT NEG	+++PT NEG	++++T NEG	++---- NEG	PPT-- NEG	+P---	"	T----	"
926 I. V.	+P--- NEG	+PT-- NEG	+PTT- POS	++PT- POS	++PT- NEG	++++- NEG	+PT NEG	+P--- NEG	PPT--	"	++++P	POS. ABSCESS
943 S. C.	++P-- NEG	++PT- POS	+PT-- POS	++PT- NEG	++PT- NEG	++---- NEG	+PT-- NEG	TT--- NEG	PT---	"	PPT--	NEGATIVE
955 S. C.	+P--- NEG	+++PT NEG	++++P NEG	+++PT NEG	++++P NEG	++P-- NEG	P---- NEG	P---- NEG	+P---	"	NOT DONE	"
972 S. C.	PT--- NEG	PT--- NEG	TT--- NEG	++P-- NEG	+PT-- NEG	++P-- NEG	+PT-- NEG	+P--- NEG	-----	"	++T--	"
968 S. C.	++PT- NEG	+++PT POS	++PT- NEG	++++P NEG	+++PT NEG	+PT-- NEG	+PT-- NEG	++TT- NEG	+PT--	"	NOT DONE	"
948 S. C.	++P-- NEG	+++PT POS	+++PT NEG	++++P NEG	+++++ NEG	+++P- NEG	+PT-- NEG	PTT-- NEG	+P---	"	+++++	"
912 S. C.	++PT- NEG	+++PT NEG	++PT- NEG	++++P NEG	++P-- NEG	++++P NEG	+PT-- NEG	+P--- POS	PT---	"	++PT-	"
944 S. C.	+P--- NEG	+++PT NEG	++PT- NEG	+++PT NEG	+++PT NEG	+++P- NEG	+PT-- NEG	+PT-- NEG	+TT--	"	+++PT	"
940 S. C.	++PT- NEG	++++P NEG	++++P NEG	++++P NEG	+++PT NEG	NOT DONE	+T--- NEG	PPT-- NEG		"	++++P	"

+ = Complete agglutination; P = Partial agglutination; T = Trace of agglutination; — = No agglutination.

Normal, indicates the litter was normal, and the cultures from fetal membranes, milk, and destroyed or dead pigs were negative for *Br. suis*.

Pos. = *Br. suis* isolated from the blood stream.

Neg. = *Br. suis* was not isolated from the blood stream.

groups were conducted at intervals of approximately one week for three months and approximately two weeks thereafter for two months. Serum agglutination tests were conducted at intervals corresponding to those of the blood cultures.

Bacteriological examinations were made of the tissues of some pigs from nearly every litter and from placentae and colostrum in all seven groups of swine. In addition, 7 gilts and 8 sows were destroyed during the course of the experiments for bacteriological and pathological examinations. Both the direct culture and guinea-pig inoculation methods were used in these examinations. Direct cultures were made, using aseptic technique and tryptose agar plates. The guinea pigs inoculated with tissues or colostrum were destroyed for bacteriological examinations after an incubation period of approximately one month.

RESULTS

In table 1 are set forth the detailed results of the subcutaneous or intravenous reëxposure of 15 sows in group 1 (*a*), all of which had been exposed as weanling pigs as follows: 4 intravenously, 3 subcutaneously, 2 *per os*, 3 *per vagina*, 2 *per conjunctiva*, and 1 *per rectum*. All but 3 of these sows (947, 943, 972) developed a higher agglutination titer after reëxposure than they were showing immediately prior thereto. Nine of the sows yielded positive bacteriological findings for *Br. suis* from the blood one or more times subsequent to reëxposure. All of the sows farrowed full-term litters except one (903) which failed to conceive. Bacteriological findings at parturition were negative for *Br. suis* in all cases. Colostrum agglutination titers from 11 sows were relatively high in 8 cases and negative or low in 3 cases. Colostrum was not obtained from 4 sows. Three sows (940, 926, 947) were destroyed for bacteriological examination for *Br. suis* five, twelve, and thirteen months after reexposure, respectively. *Br. suis* was isolated from a large abdominal abscess of sow 926. The other 2 sows were negative for *Br suis* cultures.

The detailed results of the subcutaneous or intravenous reëxposure of 4 sows in group 1(*b*), all of which had failed to become infected when exposed to *Br. suis* as weanling pigs, are presented in table 2. Two sows (907 and 909) developed a marked rise in agglutination titer, and *Br. suis* cultures were obtained from their blood following reëxposure to *Br. suis*. Two other sows (914 and 920) did not respond to reexposure with any marked increase in agglutination titer but did develop a bactere-

Table 2—Results of the Subcutaneous and Intravenous Reëxposure of 4 Sows in Group I (b)

SOW NO. ROUTE RE-EXP	PRE-RE-EXP TITER	AGGLUTINATION TITERS AND BLOOD CULTURAL RESULTS TIME IN DAYS AFTER RE-EXPOSURE 14	29	41	55	70	101	139	202	FARROWING RESULTS	COLOSTRUM TITER	BR. SUIS CULTURES AT PARTURITION OR AUTOPSY
907 I.V.	+PT-- NEG	+++++ POS	+++++ POS	+++++ NEG	+++++ NEG	+++P NEG	++PT NEG	PPTT- NEG	++PTT	NORMAL	+++PT	POSITIVE CULTURES FROM COLOSTRUM
909 S.C.	++P-- NEG	++++P POS	+++++ NEG	+++PT POS	++++P POS	+++PT NEG	++PT- NEG	++PT- NEG	++TT-	,,	+++PT	NEGATIVE
914 S.C.	++--- NEG	++PT- POS	+PT-- POS	+PT-- NEG	+PP-- NEG	+PT-- NEG	PT--- NEG	+PT-- NEG	+PT	,,	+++PT	,,
920 S.C.	+PT-- NEG	++PT- NEG	++PT- NEG	++PT POS	NOT DONE	++PT- NEG				,,	NOT DONE	,,

+ = Complete agglutination; P = Partial agglutination; T = Trace of agglutination; — = No agglutination.

Normal, indicates the litter was normal, and the cultures from fetal membranes, milk, and destroyed or dead pigs were negative for *Br. suis*.

Pos. = *Br. suis* isolated from the blood stream.

Neg. = *Br. suis* was not isolated from the blood stream.

Table 3—Results of Exposure of 3 Sows to Brucella Suis per Os, Subcutaneously, and Intravenously

SOW NO	PRE-EXP TITER & BLOOD CULTURE	EXP. ROUTES	AGGLUTINATION TITERS AND BLOOD CULTURAL RESULTS TIME IN DAYS AFTER EXPOSURE 14	29	41	55	70	101	139	202	FARROWING RESULTS	COLOSTRUM TITER	BR. SUIS CULTURES AT PARTURITION OR AUTOPSY
945	PT---	ORUM	T----	+PT--	++P-- POS.	++PT-	+PT--	+PT--	PT---		NORMAL	++PPT	NEG.
946	T----	SUBCUT.	+++++	+++++	++++P POS.	++++P	+++PT	+++PT	++PT-	PPT---	,,	+PT--	,,
964	PT---	INTRA-VENOUS	+++++ POS.	++++P		++PT-	++++P	+++--					POS. UTERUS & ING. LYMPH NODE

+ = Complete agglutination; P = Partial agglutination; T = Trace of agglutination; — = No agglutination.

Normal, indicates the litter was normal, and the cultures from fetal membranes, milk, and destroyed or dead pigs were negative for *Br. suis*.

Pos. = *Br. suis* isolated from the blood stream.

Blood culture examinations not recorded as positive were negative for *Br. suis*.

Table 4—Results of Exposure of 12 Yearling Gilts to Brucella Suis, per Os and Vagina

SOW NO	PRE-EXP TITER & BLOOD CULTURE	EXP ROUTES	AGGLUTINATION TITERS AND BLOOD CULTURAL RESULTS TIME IN DAYS AFTER EXPOSURE 14	29	41	55	70	101	139	202	FARROWING RESULTS	COLOSTRUM TITER	BR. SUIS CULTURES AT PARTURITION OR AUTOPSY
559	P----	ORUM VAGINA	+++++ POS.	++PT-	+PT--	+PT--	++PT-	+PT--	+P---	+PT--			
485	T----	,,	++P--	++P--	+PT--	PT---	++P--	++PT- POS.	+PT--	+P---	NORMAL	+++PT	POS. COLOSTRUM
560	T----	,,	++PT- POS.	++P--	++PT-	+PT--	+P---	+PT--	PT---		,,	+++PT	NEG.
487	-----	,,	++PT- POS.	+++PT POS.	++++P	+++PT	+++PP	++PT-	++P--	PT---	,,	+++TT	,,
583	-----	,,	+++PT POS.	+++--	+++PT POS.	+++++ POS.	+++PT	+++PT POS.	+++PT	+PT--	,,		POS. COLOSTRUM & PLACENTA
489	-----	,,	++PT- POS.	+PT--	PPT--	PT---	++---	+PT	+P---		,,	-----	NEG.
490	T----	,,	++PT- POS.	+P---	+PTT-	+PTT-	PT---		T----		,,		,,
491	T----	,,	+++--	++T--	+++PT	++PT-	++---	T----	T----	PT---	,,	++PT-	POS. LYMPH NODES
492	+PT--	,,	++PT-	+PT-- POS.	+++PT POS.	+++++ POS.	++++P	+++++	+++++	+++++	,,		POS. COLOSTRUM
558	-----	,,	+PTT- POS.	+++PT	+++PT POS.	+++PT	+++PT-	+++PT POS.	+++T	++T--	,,	+++PT	POS. COLOSTRUM
494	T----	,,	++PT-	++T--	++PT-	+++TT POS.		+PT-- POS.	++P--		,,		POS. HEPATIC NODE
495	T----	,,	++PT-	++PT-	+++PT	+PT--		+P-T-	+P---		,,		NEG.

+ = Complete agglutination; P = Partial agglutination; T = Trace of agglutination; — = No agglutination.

Normal, indicates the litter was normal, and the cultures from fetal membranes, milk, and destroyed or dead pigs were negative for *Br. suis*.

Pos. = *Br. suis* isolated from the blood stream.

Blood culture examinations not recorded as positive were negative for *Br. suis*.

mia resulting in the isolation of *Br. suis* from their blood. Three of these 4 sows farrowed full-term litters with negative bacteriological findings at the time of parturition. Such examinations included the tissues cultured from 1 sow (920) which was destroyed the day following parturition. The fourth sow in this group (907) eliminated *Br. suis* in the colostrum following her second pregnancy and aborted in the third pregnancy at fifty-seven days. Three of these sows showed a relatively high colostrum titer. Colostrum was not obtained from the fourth sow.

Table 3 shows the results of exposing the 3 unexposed sows in group 2 (945, 946, 964) to *Br. suis per os*, subcutaneously, and intravenously, respectively, *i.e.*, one exposure per sow. These 3 sows had no previous history of exposure to *Br. suis* and were known to have maintained negative agglutination titers and blood cultures since they were weanling pigs. Following exposure, all 3 developed positive agglutination titers, and *Br. suis* was isolated from the blood stream of each sow at intervals. Two sows (945 and 946) farrowed normally, and *Br. suis* was not recovered at the time of parturition. Both of these sows had positive colostrum titers. One sow (945) was destroyed for purposes of examination six months after exposure, and bacteriological examination of her tissues was negative for *Br. suis*. Another sow (964) in this group was destroyed three months after exposure, and *Br. suis* was isolated from the superficial inguinal lymph nodes and uterus. This sow was in advanced pregnancy when destroyed. Sow 946 was retained for further observation.

Table 4 presents the detailed data resulting from the exposure of the 12 unexposed yearling gilts in group 3 to *Br. suis per os* and *per vagina*. All of these gilts subsequently developed relatively high agglutination titers and 10 of the 12 developed a bacteremia, as evidenced by the isolation of *Br. suis* from their blood. All but 1 gilt (559) farrowed full-term litters. Gilt 559 did not conceive. Four of the gilts eliminated *Br. suis* in the colostrum at parturition, and the placenta of 1 of the 4 was infected. Positive bacteriological findings for *Br. suis* were obtained from the lymph nodes of 2 gilts (494 and 491) which were destroyed for purposes of examination six and twelve months, respectively, after exposure. Two other gilts (492 and 583) yielded no positive cultures for *Br. suis* when destroyed twelve months after exposure.

The detailed results of exposing the 6 unexposed yearling gilts in group 4 to *Br. suis per conjunctiva, os*, and *vagina* are

Table 5—Results of Exposure of 6 Yearling Gilts to Brucella Suis, per Os, Conjunctiva, and Vagina

SOW NO.	PRE-EXP. TITER & BLOOD CULTURE	EXP. ROUTES	AGGLUTINATION TITERS AND BLOOD CULTURAL RESULTS TIME IN DAYS AFTER EXPOSURE 33	71	134	FARROWING RESULTS	COLOSTRUM TITER	BR. SUIS CULTURES AT PARTURITION OR AUTOPSY
28	-----	ORUM VAGINA CONJ.	PT--- POS.	T----	PT---	NORMAL	+++PT	NEG.
46	T----	"	PT---	TT---	T----	"	+P---	"
50	T----	"	++P-- POS.	+++PT	++++P			
871	PT---	"	+P---	+PP--	+P---	ABORTED		POS FETI & PLACENTA
55	-----	"	+P---	++P--		NORMAL		NEG.
30	-----	"	PT---	T----				POS. MANDIBULAR LYMPH NODE

+ = Complete agglutination; P = Partial agglutination; T = Trace of agglutination; — = No agglutination.

Normal, indicates the litter was normal, and the cultures from fetal membranes, milk, and destroyed or dead pigs were negative for *Br. suis*.

Pos. = *Br. suis* isolated from the blood stream.

Blood culture examinations not recorded as positive were negative for *Br. suis*.

Table 6—Results of Feeding Aborted Materials to 5 Previously, Naturally Infected Sows

SOW NO	PRE-RE-EXP. TITER & BLOOD CULTURE	AGGLUTINATION TITERS AND BLOOD CULTURAL RESULTS TIME IN DAYS AFTER RE-EXPOSURE 6	13	20	28	34	41	48	55	62	71	85	99	110	127	155	BR. SUIS CULTURES AT PARTURITION OR AUTOPSY AND FARROWING RESULTS
808	-----	PT--	P---	T---	PT---	PT---	PT---	T---	T---			-----	++PT-	+PPT-	+PT--	+PT--	POS. ABSCESS
815	+PT--	+PT--	+++T-	PT--	++PTT	+PT--	+PT--	+P--	PPPT-			+PTT-	++PT-	++PT-	+++PT	+++PT	NORMAL
810	T----	+PT--	PPT--	+PT--	+PTT- POS.	++PTT	+++PT	+++P-	+PT--	++PTT	++P-T	+PT--	++PTT	++PT-			NOT PREGNANT
827	-----	PT---	TT--	+PPT-	PT---	+PTT-	+P---	++P--	PT---	+PT--	+PT--	+PT--	+PT--	PT---			" "
832	PT---	+PT--	PT---	PT--- POS.	PT---	PTT--	PT---	PT---	TT---	+PT-- *	+PT--	PTT--	+PTT-	PT--- POS.	+PT--	+PT--	" "

* SECOND RE-EXPOSURE GIVEN PRIOR TO THIS TEST.

+ = Complete agglutination; P = Partial agglutination; T = Trace of agglutination; — = No agglutination.

Normal, indicates the litter was normal, and the cultures from fetal membranes, milk, and destroyed or dead pigs were negative for *Br. suis*.

Pos. = *Br. suis* isolated from the blood stream.

Blood culture examinations not recorded as positive were negative for *Br. suis*.

Table 7—Results of Feeding Aborted Materials to 6 Second-Pregnancy Sows, Exposed Previously to Brucella Suis in Their First Pregnancy

SOW NO	PRE-RE-EXP. TITER & BLOOD CULTURE	AGGLUTINATION TITERS AND BLOOD CULTURAL RESULTS TIME IN DAYS AFTER RE-EXPOSURE 6	13	20	28	34	41	48	55	62	71	85	99	110	BR. SUIS CULTURES AT PARTURITION OR AUTOPSY AND FARROWING RESULTS
46	T----	+T---	PT---	PT---	T----	P----	+P---	PT---	PT---	T----	T----	T----		T----	NORMAL
441	+++PT	+++++	+++++	++++P	+++PT	+++++	+++++	++++-	+++++	+++++	+++++	+++PT	+++++	++++P	NOT PREGNANT
594	-----	-----	-----	T---- POS.	+PT--	++PT- POS.	++PT-	+PT--	++PT-	++PT-	++P--	+++PT	T----	+PT--	NORMAL
28	+P---	+PT--	P----	PT---	PT--- POS.	+++PT	+++T- POS.	++TT-	+PT--	++PT-	++PT-	+PT--	+PT--	++PT-	NORMAL
558	++++P	+++PT	+++PT	+PT--	++PT-	++PTT	++PT-		++PPT	++PPT	+++PT POS.		+++PT POS.	+++PT	NORMAL
587	-----	-----	-----	T----	P----	+P---	++T--	T----	PT---	T----	PT---	PT---	PT---	PT---	NORMAL

+ = Complete agglutination; P = Partial agglutination; T = Trace of agglutination; — = No agglutination.

Normal, indicates the litter was normal, and the cultures from fetal membranes, milk, and destroyed or dead pigs were negative for *Br. suis*.

Pos. = *Br. suis* isolated from the blood stream.

Blood culture examinations not recorded as positive were negative for *Br. suis*.

shown in table 5. The gilts were exposed on or near the day of breeding.

With the exception of 1 gilt (50), relatively high agglutination titers were not demonstrated. Two of these gilts (28 and 50) yielded positive bacteriological findings for *Br. suis* from the blood. Two gilts (30 and 50) failed to farrow, but since they were not observed to show regular estrual cycles, it is possible that they may have conceived and experienced early, unobserved abortions. One gilt (871) aborted twenty-three days after exposure, and *Br. suis* was isolated from the fetuses and fetal membranes. This gilt continued to eliminate *Br. suis* in the discharges from the genital tract for a period of at least fifty-one days following the abortion and never developed an agglutination titer greater than incomplete at 1:100. The remaining 3 gilts (28, 46, 55) in this group farrowed small, full-term litters of 7, 2, and 1 pig, respectively, with negative bacteriological findings for *Br. suis* at the time of farrowing. Two gilts (30 and 55) were destroyed for purposes of bacteriological examination three and one-half and twelve months, respectively, after exposure. *Br. suis* was isolated from the mandibular lymph nodes of sow 30.

In table 6 the detailed results are set forth of the feeding of aborted materials to the 5 sows in group 5, which had previously been infected under natural field conditions. Only 2 of these sows (808 and 815) were pregnant at the time of reëxposure. No abortions were observed. Two sows (810 and 832) became reinfected as was evidenced by the isolation of *Br. suis* from their blood and the increased agglutination titers. One sow (808) developed a large abscess on the right thoracic wall after reëxposure. This swelling was allowed to remain for a long period of time. On Jan. 11, 1945, pus was withdrawn from this abscess and *Br. suis* was isolated from this pus by both direct culture and guinea-pig inoculation. The agglutination titer of this sow at the time of such findings was complete at 1:50 only. Two sows (827 and 815) showed increases in agglutination titers, but *Br. suis* was not recovered from either of these sows. Sow 832 was reëxposed by feeding aborted materials a second time, sixty-two days later and developed an additional increase in agglutination titer, and *Br. suis* was again isolated from the blood of this sow.

The results of feeding aborted materials to the 6 second-pregnancy sows in group 6, previously exposed to *Br. suis* in their first pregnancy, are presented in table 7. Three sows (28, 558, 594) became reinfected as evidenced by the isolation of *Br. suis* from their blood stream and increased agglutination response. All but 1 of the sows (441) farrowed normally. Sow 441 was not pregnant.

Table 8 presents the results of feeding aborted materials to the 5 negative control sows in group 7. Two sows (340 and 344) aborted at fifty-eight and fifty-three days, respectively. The other 3 sows (369, 348, 329) either did not conceive or aborted unnoticed. All 5 sows developed positive agglutination titers, and *Br. suis* was recovered from their blood streams. *It is most interesting to note that Br. suis was recovered from 2 sows (329 and 369) six days after exposure per os, and all 5 sows yielded positive blood cultures for Br. suis by the twentieth day after exposure. Br. suis* was isolated from the tissues of 2 sows (340 and 348, at autopsy.

DISCUSSION

In this report are included the records of experiments with seven separate groups of sows and gilts, with a total of 56 animals artificially exposed and/or reëxposed to *Br. suis*. It is necessary to consider the objectives for each group in order to more intelligently discuss and interpret the results.

Group 1 consisted of 19 second-pregnancy sows which were reëxposed to *Br. suis* for the purpose of challenging their degree of resistance or immunity which might have resulted from a previous artificial exposure at weaning age. These sows had carried their first litters without a single instance of abortion and without the isolation of Brucella from any of them at the time of their first parturitions. Therefore, it was

Table 8—Results of Feeding Aborted Materials to 5 Brucella-Free Sows

SOW NO.	PRE-EXP. TITER & BLOOD CULTURE	AGGLUTINATION TITERS AND BLOOD CULTURAL RESULTS TIME IN DAYS AFTER EXPOSURE															BR. SUIS CULTURES AT PARTURITION OR AUTOPSY AND FARROWING RESULTS
		6	13	20	28	34	41	48	55	62	71	85	99	110	127	155	
340	-----	-----	+P--- POS.	PTT-- POS.	+++++ POS.	++PTT POS.	+++++		+++PT POS.		DESTROYED 6-7-44 POSITIVE CULTURES FROM LIVER, LUNG, SPLEEN, & LYMPH NODES.						ABORTED, 58 DAYS POSITIVE MEMBRANES & PIG FETI.
344	-----	-----	++P-- POS.	+++PT	+++PT POS.	+++PP POS.	++PPT	+++PT	+++PT		+++++	+++++P	+++++	+++PT	+++PT	+++PT	ABORTED, 53 DAYS POSITIVE MEMBRANES & PIG FETI
369	-----	T---- POS.	++PT- POS.	+++PT POS.	++++P POS.	+++PP POS.	+++PT POS.	+++PT	+++PT POS.		+++++	+++++	++++P	+++++	+++++	+++++	NOT PREGNANT
348	-----	-----	PT---	+PT-- POS.	+++PT POS.	+++PT POS.	++PT- POS.	+++PT POS.	++PT- POS.		DESTROYED 7-29-44 POSITIVE CULTURES FROM UTERUS, OVARIES, & COMPOSITE AUTOPSY TISSUES						NOT PREGNANT
329	T----	+T--- POS.	+P--- POS.	++PT- POS.	+++PT POS.	+++PT POS.	+++PT POS.	+++PT POS.	+PT-- POS.	+++PT	++PT-	+++PT		+++++			NOT PREGNANT

+ = Complete agglutination; P = Partial agglutination; T = Trace of agglutination; — = No agglutination.

Pos. = *Br. suis* isolated from the blood stream.

Blood culture examinations not recorded as positive were negative for *Br. suis*.

reasonable to assume that they might have developed a considerable degree of immunity as a result of their exposure at weaning age. Under these conditions, it seemed desirable to challenge their possible immunity by injecting viable *Br. suis* intravenously and/or subcutaneously, rather than to employ natural channels of infection for such tests for immunity. In other words, the objective in this case dictated that such severe reëxposure be employed, thus eliminating chance and variable factors of reëxposure.

In general, these sows became reinfected under these conditions as measured by positive *Br. suis* cultures from their blood streams and increases in blood agglutination titers. Also, in 1 case, *Br. suis* was recovered at the time of parturition. In no case did this type of reëxposure result in abortions. Thus it appears that the degree of immunity in these sows was not sufficiently marked to protect against the severe type of reëxposure employed in these experiments.

The objectives with groups 2, 3, and 4, included in this report, were to produce abortions if possible and to study the course of this disease in these age groups of swine following artificial exposure with *Br. suis*. In other words, we had to consider the possibility that even the guinea-pig tested polyvalent cultures employed as an inoculum in these and previous experiments were not capable of regularly causing exposed swine to abort. It seemed likely that our knowledge of channels of exposure or other factors, such as the stage of gestation at the time of exposure, was inadequate to regularly produce abortions experimentally in sows. All of these questions were not satisfactorily answered by these experiments, because abortions were not regularly produced in these animals by these methods of artificial exposure. However, the results in these three groups of sows do serve as a check on the pathogenicity for hogs of the cultures employed as an inoculum for challenging the degree of immunity in the first group of sows, because they demonstrated that susceptible swine did develop a syndrome following exposure with these cultures. The syndrome developed included abortion after twenty-three days in 1 gilt and the development of positive serum agglutination reactions and a bacteremia and localization of the organisms in lymphatic tissues of some of the experimental swine. These results, from the standpoint of the syndrome produced by artificial exposure of swine, are consistent with those reported in natural outbreaks of the disease by Johnson and

Huddleson[4] and also with some of our observations on natural outbreaks in the field.

It is worthy of note, that despite these results in which abortions were not regularly produced by these experimental methods, the evidence is that the cultures employed as inoculum were pathogenic for man. Both of two barn men taking care of these experimental swine became ill with undulant fever in spite of all our efforts to prevent such infection. These methods of preventing infection in workers have been previously described.[5] Thus, we conclude that these cultures were pathogenic for both swine and man.

The failure of the gilts and sows in groups 2, 3, and 4 to abort regularly following exposure to polyvalent inoculum of *Br. suis* cultures may be associated with the stage of pregnancy at the time of exposure. Our observations of natural outbreaks of brucellosis in swine suggest that abortions are more apt to result when exposure occurs at or near the time of breeding and by service from an infected boar. It is of interest to note that in the exposures of the latter three groups the only observed abortion occurred in a gilt that was exposed two days following breeding.

Groups 5 *and* 6 were reëxposed to *Br. suis* by feeding aborted material for the purpose of challenging their degree of resistance or immunity which might have resulted from a previous natural or artificial exposure during adult or sexually mature life and also to check the previous results obtained with the sows in group 1.

The results show that of 11 sows (5 naturally infected and 6 artificially infected previously in adult life) 6 became reinfected when reëxposed *per os* to aborted fetuses and membranes. Also, in 1 case, a naturally infected sow (832) was twice reëxposed and twice developed an increased agglutination response combined with positive blood cultures for *Br. suis* after each reëxposure. In no case did these reëxposures result in abortion. Thus we conclude that although some tolerance was developed, there was not sufficient immunity in these sows to protect them against this more natural type of reëxposure.

Group 7, composed of five negative sows, was exposed to *Br. suis* by feeding aborted materials for the purpose of comparing the results of such exposure to those obtained with groups 5 and 6, to produce abortion if possible and to study the course of this disease in these swine. It will be noted that these negative control sows present a much more vivid picture of infection than the reëxposed sows in groups 5 and 6. This again indicates that there is some degree of resistance present in swine previously exposed to *Br. suis*, but it is not sufficient to class them as immune.

Considering only the symptom of abortion, the farmer and some others might assume that the sows in this experiment, which had been exposed as weanling pigs and reëxposed in their second pregnancies, were immune. However, if the rigid and more correct criterion of active infection is applied, it is evident that some of these animals did become reinfected, were not immune, and would constitute a possible menace to other swine, cattle, and human health. Consistent with this view, Huddleson,[6] in reviewing active immunity developed against Brucella infections by cattle, states, "The only sure indicator to active immunity to brucellosis is resistance to infection." Thus it is not sufficient to attempt to measure the effects of brucellosis in any species merely in terms of the percentage of observed abortions.

SUMMARY

1) Of 19 sows previously exposed to *Brucella suis* as weanling pigs and reëxposed by intravenous or subcutaneous injections during their second pregnancies, 13 became reinfected as evidenced by increases in agglutination titers and recovery of *Br. suis* from their blood streams. No abortions were observed in this group; however, 1 of these sows aborted at fifty-seven days in her third pregnancy, and *Br. sius* was isolated from the aborted pigs, membranes, and uterine discharges.

2) Twelve yearling gilts and 3 second-pregnancy sows, not previously exposed to *Br. suis* but kept as controls, were exposed

to the same cultures on the same day as the 19 previously exposed sows. Of these controls, *Br. suis* was isolated from the blood streams of 10 gilts and 3 sows, and all 15 developed positive serum agglutination titers, but none aborted.

3) In addition, the 6 brucellosis-free yearling gilts which were exposed to transplants of the same cultures two months later at or near breeding time became infected, and 1 aborted, while 2 others may have had unobserved abortions since they failed to farrow.

4) Of 5 previously naturally infected sows, 3 became reinfected when fed infected aborted materials as the source of reexposure.

5) Six sows previously exposed to *Br. suis* during their first pregnancy were reexposed to *Br. suis* by feeding small portions of aborted materials after their second breeding. *Br. suis* was isolated from the blood of 3 sows, and increased agglutination titers were observed in 5 of the sows.

6) Of 5 brucellosis-free yearling gilts used as controls for this reëxposure feeding experiment, all became infected, 2 aborted, and *Br. suis* was recovered from the aborted pigs and membranes.

7) In these experiments the hogs previously exposed to *Br. suis* by either natural or artificial means were not as responsive to a second exposure as unexposed swine of comparable ages were to their first exposure, but the resistance or tolerance in these previously exposed animals was not sufficient to prevent reinfection.

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Brucellosis of Swine

IV. The Unit-Segregation System of Eradication

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THIS PAPER describes a unit-segregation system of eradicating swine brucellosis, under California conditions. The data presented have been compiled entirely from the swine herd of a state hospital. Although 19 additional herds are included in the station project on this disease, the hospital herd is selected as the specimen because of its size, the completeness of data, the manager's liberal coöperation, and the difficulties involved in bringing the program to a successful conclusion. A preliminary account of the hospital swine (herd 1) was given by Cameron and Carlson.[1] Though infection was still present at the time of that report, evidence from the herd, as well as from others, suggested that a unit-segregation system would operate successfully in eradication. For the purpose of this work, the unit is defined as a group segregated on the basis of their exposure to brucellosis, not on the basis of their individual reactions to the test. A positive unit contains reactors of breeding age, and also negative animals of that age that have been exposed to infection by associating with reactors. The negative unit contains no animals showing a titer higher than 1:25. If infection exists within a herd, as demonstrated by the agglutination test, the entire herd is considered infected and constitutes the positive unit. A negative unit is formed from the progeny of the positive unit, and segregated after weaning. At no time are the units allowed to intermingle. As replacements become available, the positive unit is culled, and, as the following data will show, certain valuable individuals can be salvaged at that time.

From the Division of Veterinary Science, University of California, Davis.
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According to Hutchings, Delez, and Donham[2] and Cameron and Carlson[3], the agglutination test is not a reliable means of diagnosing brucellosis in the individual pig. To enhance the effectiveness of the test, Cameron and Carlson[3] tried lowering the serum dilution to 1:6.25. They concluded that the modification did not increase the effectiveness of the test in picking out non-infected animals from an infected herd. Besides positive hemocultures at the lower dilution in experimental exposure, unpublished data from these workers show, in field investigations, gilts negative at 1:6.25 reacting at 1:200, two weeks later; some of these aborted while still negative. Though the inefficiency of the test can be attributed partly to the period of incubation, it must also result from an inherent variation in individual susceptibility. Judging from the work of Cameron, Gregory, and Hughes[4] in a bacteriological examination of resistant stock and the studies of Cameron and Carlson[3] on the diagnostic titer, certain individuals may be temporarily infected without reacting to the test. Whether, during the transitory infection, such animals shed the organism depends on their status with respect to pregnancy. On the other hand, the test effectively determines the presence of infection within a group of swine. Obviously, therefore, a blood-testing program aiming at eradication must be based on the reaction of the unit as a whole, not of the individual.

Usually, as Cameron and Carlson[1] have shown, weaned pigs are not infected even if weaned from reacting sows. Though they are susceptible, if segregated they will not only remain negative, but will prevent reinfection in the positive unit. The data presented here, therefore, will show (1)

further evidence on the freedom of weaned pigs from the infection, (2) recovery in a segregated positive unit, and (3) the duration of titers in a segregated positive unit.

TABLE 1—Results of Tests on Progeny Weaned and Segregated Immediately from Positive Unit

Time of Farrowing	Date of Testing Progeny	Number of Progeny				
		Tested	Negative	Reacting to Titers of 1:25	1:50	1:100
Spring '43	8/25/43	59	58	0	0	1*
	10/13/43	48	48	0	0	0
	11/18/43	106	106	0	0	0
Summer '43	1/19/44	24	24	0	0	0
	2/ 9/44	22	22	0	0	0
Fall '43 and Spring '44	10/ 6/44	40	38	0	0	2
	1/30/45	32	31	0	0	1
	3/ 7/45	31	31	0	0	0
Fall '44	2/28/45	29	29	0	0	0
	3/28/45	24	21	2	1	0
Total tests		415	408	2	1	4
Total pigs		200	...	2	1	4

*Positive lymph gland culture.

Results

Table 1 gives the results of testing the progeny of the positive unit periodically between weaning and breeding. Clearly, no spread of infection that could be demonstrated by the test occurred in these open young gilts. The presence of latent infection rather than reinfection is suggested in the results; exposure would have been reflected in a large number of reactors, as occurred in earlier litters noted in the preliminary report.[1]

When a segregated positive unit had been established, the trend in titers could be studied in a group of sows that had gone through an abortion storm, were now reproducing normally, and were not actively spreading the infection to susceptible gilts. (In the latter, abortion may occur, with resultant reinfection of sows.) Table 2 shows the results of periodic testing in the unit. Evidently, the sow has a marked ability to recover from the infection. The time of exposure or initial reaction was not available in this group, since these animals represent the purchased foundation stock of the herd and were infected when purchased. Because of incomplete segregation, infection spread in the first progeny from the foundation stock before they reached breeding age. Data are available, therefore, on the duration of titers in a group of gilts exposed naturally to *Brucella suis* at an age of approximately 6 months. Table 3, the complete protocols on this group, shows the trend in titer from initial reaction until recovery. According to the statistical summary of the results (table 4), recovery may be expected in a comparable group at approximately seven months provided they are not exposed to reinfection.

Discussion

The basis of the unit-segregation system is, first, the efficiency of the test when applied to a group; second, the breaking of the chain of infection from infected sow to bred gilt, and from aborting gilt back to the resistant but potential spreader sow. In California, bred gilts are usually placed with the bred sows. The latter, if infected, transmit brucellosis to the gilts, which, being susceptible, are apt to abort and thus reinfect the sows that otherwise may have recovered. Although a reacting sow may shed the organism at farrowing, she is invariably confined to a separate pen at the time and, therefore, not a menace to others in the unit.

The foregoing data are further evidence on the resistance of weanling pigs to natural exposure and on the feasibility of making such animals the nucleus of a clean unit. In pigs weaned from infected sows, a little residual or latent infection may per-

TABLE 2—Agglutination Reactions in the Foundation Gilts to Titers of 1:25, 1:50, 1:100, and 1:200 on the Designated Dates

Sow	7/29/42	11/18/43	1/7/44	2/23/44	6/12/44	2/21/45
1	+ + + +	— — — —	— — — —	— — — —		I — — —
2	+ + + +	+ + + +	+ + + +			
3	+ — — —	— — — —	— — — —	— — — —		— — — —
4	+ + + +	— — — —	— — — —	— — — —	+ — — —	— — — —
5	+ + + —	— — — —	— — — —	— — — —	— — — —	— — — —
6	+ + — —	— — — —	— — — —	— — — —		— — — —
7	— — — —		— — — —	— — — —		— — — —
8	+ + — —	I — — —	— — — —	— — — —		
9	+ + + +	— — — —				
12	+ + + +		— — — —	— — — —		— — — —
14		+ — — —	— — — —	I — — —		
15	+ + + +	— — — —	+ — — —		— — — —	— — — —
16	— — — —	— — — —	— — — —	— — — —		— — — —
17	+ I — —	— — — —	— — — —	— — — —		— — — —
18	+ — — —	— — — —	— — — —	I — — —		
19	+ + + —	— — — —	— — — —	I — — —		— — — —
20	+ + + I	+ I — —	+ + — —		— — — —	— — — —
21		+ + I —	— — — —	+ + I —	I — — —	— — — —
22	— — — —	— — — —	— — — —	— — — —		
23		— — — —	— — — —	+ I — —	I — — —	— — — —
24	— — — —	— — — —	— — — —		— — — —	— — — —
25	+ + + +		— — — —	— — — —	— — — —	— — — —
26	+ + + +	— — — —	— — — —	— — — —		
27	+ + + +	+ + I —	+ + — —			I — — —
30		— — — —	— — — —	— — — —		— — — —
31	— — — —	— — — —	Autopsy culture neg.			
32	— — — —	— — — —				
33	— — — —	+ — — —	— — — —		— — — —	— — — —
34			— — — —	— — — —		— — — —
35	— — — —		— — — —	— — — —		— — — —
36		— — — —	— — — —	— — — —		— — — —
37		— — — —	Autopsy culture neg.			
39	+ + + I	— — — —	— — — —		— — — —	— — — —
40	— — — —	— — — —	— — — —	— — — —		
41	— — — —		— — — —			
42	— — — —	— — — —	— — — —	— — — —		+ — — —
43	+ + + +	— — — —	— — — —	— — — —		— — — —
44	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —
45	+ + + +	— — — —	— — — —	— — — —	— — — —	
46	— — — —	— — — —	Autopsy culture neg.			
47	+ + + +	— — — —	— — — —	— — — —		
48		I — — —	— — — —	+ I — —		
49	+ + — —		— — — —	— — — —		— — — —
50	+ + — —		+ + I —			
52	+ + + +	Autopsy culture neg. 9/10/43				
53	+ I — —	— — — —	— — — —	— — — —		— — — —
54	+ + + +	— — — —	— — — —	— — — —		— — — —
55	+ + — —	— — — —	— — — —	— — — —	— — — —	— — — —
56	+ + + +	— — — —	— — — —			
57	+ + + +	+ + + +	+ + + +			
58	— — — —	+ + — —	+ + + +		+ I — —	+ + — —
59	+ + + +	— — — —	— — — —		— — — —	
60	+ + + +	+ I — —	+ I — —			— — — —
62	+ + + +	+ + — —	+ + — —		+ + — —	+ — — —
63	+ + + +	+ + + —	+ + I —			
64	+ + + +	I — — —	— — — —	— — — —		
65	+ + + +	— — — —	+ — — —			
66	+ + + +	— — — —	— — — —	— — — —		— — — —
67	+ + + +	I — — —	+ — — —		+ — — —	I — — —
68	+ + — —		— — — —	— — — —		
69	+ + + +	— — — —	— — — —	— — — —	— — — —	— — — —
70	+ + + +	— — — —	I — — —		+ I — —	— — — —
71	+ + + +	— — — —	— — — —	— — — —		— — — —
72	+ + + +	— — — —	— — — —	— — — —	— — — —	— — — —
74	+ + + +	+ I — —	+ + I —		+ + — —	+ + — —
75	+ + + +	I — — —	+ — — —	— — — —		
76	+ + + +	— — — —	— — — —	— — — —		
77	+ + + +	+ + + +	+ + + +	Aborted, culture pos.		— — — —
78	— — — —	+ — — —	+ I — —		— — — —	+ + I —
79	+ + + +	— — — —	+ — — —		I — — —	— — — —
80	— — — —	— — — —	— — — —	— — — —		— — — —
81	+ + + —		I — — —			
82	+ + + +	— — — —	I — — —			
83	— — — —		— — — —	— — — —		
84	+ + + +		— — — —	— — — —		
85	+ + + +	— — — —	— — — —	— — — —		— — — —
86	+ + + +	— — — —	— — — —	— — — —		
87	+ + + —		— — — —	I — — —		— — — —
88	+ + + +	+ + + I	+ + + +		+ — — —	I — — —
89	I — — —		I — — —	— — — —		I — — —
90	+ + + —	— — — —	— — — —	— — — —		— — — —
91	+ + + +		— — — —	— — — —	+ — — —	— — — —
92	— — — —	— — — —	— — — —	— — — —	I — — —	
93	+ + + —		— — — —	— — — —		
94	+ + + I		— — — —	— — — —		— — — —
95	+ + — —	+ + + +			+ + — —	
98	+ + + +	+ + — —	+ I — —		— — — —	
99	+ + + +	— — — —	— — — —	— — — —		— — — —
100	+ + + I	— — — —	— — — —	— — — —		— — — —

TABLE 3—The Agglutination Reaction in First Progeny Following Natural Exposure in April, 1943 (Titers 1:25, 1:50, 1:100, 1:200)

Sow	Mar. 2	Apr. 5	May 10	May 28	Aug. 18	Sept., Oct.	Nov., Dec.	1944 Jan., Feb.	1945 Jan., Feb.
124	+ + + +	+ + + +			+ — — —	+ — — —	+ — — —	+ — — —	— — — —
126	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —
129	— — — —	+ + — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —
130	— — — —	+ + — —	+ + + +	+ — — —	+ + + +	+ + + +	— — — —	I — — —	— — — —
131	— — — —	— + — —	+ + + +	+ + + +	+ + + +	+ + + +	+ — — —	— — — —	— — — —
132	— — — —	— — — —	I — — —	— — — —	+ + + +	+ + + +	— — — —	— — — —	— — — —
133	— — — —	— — — —	— — — —	— — — —	I — — —	+ + + +	— — — —	— — — —	— — — —
134	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ — — —
136	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	I — — —
137	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ — — —
145	— — — —	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	I — — —
152	— + + —	+ + + —	+ + + —	+ + + —	— — — —	I I — —	I — — —	I — — —	— — — —
154	— — — —	— — — —	— — — —	— — — —	+ + + +	+ + + +	+ I — —	+ — — —	+ — — —
155	— — — —	— — — —	— — — —	— — — —	+ I + +	+ + + +	I — — —	— — — —	— — — —
156	+ I — —	+ + — —	+ + + +	+ + + +	+ + + +	+ + + +	I — — —	— — — —	— — — —
157	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	I I — —	— — — —	— — — —
163	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	I I — —	+ — — —	+ — — —
165	— — — —	— — — —	— — — —	— — — —	+ + + +	+ + + +	+ + + +	— — — —	— — — —
166	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	I — — —	— — — —
167	— — — —	— — — —	— — — —	+ + + —	— — — —	— — — —	— — — —	— — — —	— — — —
168	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —
169	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
170	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	I — — —	I — — —
171	— — — —	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	I — — —	I — — —
173	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —
174	— — — —	+ + + +	— — — —	+ + + +	+ + + +	— — — —	— — — —	— — — —	— — — —
175	— — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ I I —	— — — —	— — — —	— — — —
176	— — — —	— — — —	+ + + —	+ + + —	+ + + —	+ + + +	I — — —	— — — —	— — — —
177	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —
178	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —
181	— — — —	— — — —	— — — —	+ + + +	+ + + +	+ + + +	I I — —	I — — —	— — — —
182	— — — —	— — — —	— — — —	— — — —	— — — —	I — — —	— — — —	I — — —	— — — —
184	— — — —	— — — —	+ — — —	+ + + +	+ + + +	+ + + +	+ + + +	+ — — —	— — — —
185	— — — —	— — — —	— — — —	+ + + +	+ + + +	— — — —	— — — —	+ — — —	— — — —
186	— — — —	— — — —	+ — — —	— — — —	— — — —	+ + + +	— — — —	— — — —	— — — —
187	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	— — — —	I — — —	I — — —
189	— — — —	— — — —	+ + — —	+ + + +	I — — —	+ + + +	+ + + +	+ + + +	+ + + +

sist in individuals. Since, however, the pigs are open and, therefore, cannot spread infection, the presence of undetected infection in such a group is not serious. Cameron,[3] Gregory, and Hughes[4] were able to isolate Br. *suis* from negative open gilts up to two months after artificial exposure, but repeatedly failed to do so beyond that period. The invading organism had apparently been overcome by the resistance of the host. A blood test, therefore, of the unit prior to breeding will detect latent infection before it can be transmitted.

The data also indicate that valuable individuals may be salvaged from a positive unit when that unit is being disposed of. A residual low titer remains in many of these animals; but incomplete unpublished data, supported by the findings of McNutt,[5] indicate that they are not spreaders. Although low titers show exposure at some time or other to Brucella sp., they do not signify infection. This is especially true where repeated tests have indicated a descending titer. According to these data and those contained in the preliminary report, an animal that previously reacted at a higher dilution but which now had a 1:25 titer would be much less dangerous than one completely negative in an infected or unknown herd.

The data in table 3 corroborate the conclusion that recovery is the rule, and indicate the period when it may be expected. Conceivably, the great tendency towards recovery might be construed to indicate that the pigs could be readily immunized against Brucella sp. Present methods of immunization against brucellosis, as applied to cattle, require a living culture; the adoption of a comparable procedure for swine should not be considered until other methods have failed. In swine, the more rapid turnover and prolific reproduction that provide replacement gilts in a shorter period than in cattle are coupled with a relatively greater salvage value. For this reason, a program of test and segregation, aiming to produce a herd free from reactors, has (in California) a better chance of succeeding with swine than with cattle. The period of segregation would be short; gilts farrowed in February from a positive unit would be bred in October and November, by which time the positive unit would be farrowing another litter. When the second litter was weaned, the positive unit could be culled, since the negative would be in full production. Thus, a negative herd can be obtained in one year without financial loss; with severe culling and additional testing after weaning the first litter, the period may be reduced. Recovery will also occur in the boar, but infection may localize in the testicles. Every reacting boar, therefore, should be disposed of or, if valuable, should be isolated until his recovery is certain.

TABLE 4—Duration of Titer in 28 Gilts 6-8 Months of Age

Number of Pigs	Duration of Titer (Mo.)
4	12
6	8
3	7
9	6
5	2
1	4
2	3
1	1
Mean	6.85
Standard deviation	0.5

On most ranches, the bleeding of pigs (as compared with cattle) offers physical difficulties. A program that involves frequent bleeding has, therefore, a distinct disadvantage. The foregoing program provides for the minimum amount of testing, namely, the initial test on the herd and the test on the replacement gilts before breeding. The positive unit is bled a second time only when it is being disposed of and the owner is seeking to salvage valuable recovered sows.

California is not a large swine-breeding state and imports relatively few breeding animals. In states where the other extreme prevails and swine breeding is intensively practiced in concentrated areas, the system will perhaps prove much more difficult or even impractical. As in any other segregation plan, success depends upon the completeness of the segregation; the negative unit is a susceptible group of gilts, and the positive unit, in the first months of segregation, is a transmitting group.

SUMMARY

A unit segregation system for eradicating swine brucellosis is described. A positive unit consists of a group containing reactors, and nonreactors of breeding age that have been exposed to infection. A negative unit consists of young pigs that have been segregated from the positive unit since weaning. The system is based on the fact that young pigs are usually not infected at weaning, and on the efficiency of the test when applied to the herd.

Data are given showing the absence of infection in young pigs and on the marked tendency of pigs in the segregated positive unit to recover.

ACKNOWLEDGMENT

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Effect of Temperature, Phenol, and Crystal Violet on Vesicular Stomatitis Virus

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WITH THE KNOWLEDGE that the virus of vesicular stomatitis (V.S.) may occur in the blood of animals affected with the disease,[1,2] tests have been made to determine the effects on the virus of procedures employed in processing blood or blood serum in the preparation of biological products.

The V.S. virus used in the tests here reported was derived from the third and successive passages in guinea pigs of the M strain of New Jersey type virus[2] which was originally isolated from swine by Schoening and Crawford.[3]

Four separate heating tests were conducted in which the virus was added to Berkefeld- or Mandler-filtered serum. Serum from a hog cholera-(H.C.) susceptible pig was used in two trials and serum from a H.C.-immune hog was employed in the other two. At the time one of the latter tests was conducted, an equine and a bovine serum were included also.

Tests were conducted to determine the tenure of infectivity of V.S. virus in H.C. virus (defribrinated blood) under refrigeration (3 to 5 C.) or incubation (37.5 C.), when untreated, phenolized (0.5%), or treated with crystal violet[4,5,6] in rubber-stoppered Pyrex glass tubes.

METHODS

The hind plantar, or metatarsal, pads of guinea pigs, showing typical vesicular lesions, were removed with forceps twenty-six to fifty hours after inoculation by tunneling and pricking the skin of the hind feet with previous guinea pig-passage material. Freshly harvested virus was employed in all tests.

In the first heating test, portions of untriturated pads were placed in the serum, half of one pad being placed in the serum to be heated, the other half being placed in a control tube of serum not to be heated. Because of irregularities noted in this test, which are later referred to in this paper, subsequent tests were made with pads ground, with or without abrasive (alundum), in a mortar to which physiological salt solution was added in the quantity of 1 cc. for each pad. The finely triturated product was then strained through several thin layers of cotton or, in some cases, it was centrifuged at 500 r.p.m. or less for a few minutes to throw down the larger particles and the abrasive, when used.

A sufficient number of sterile, cotton-stoppered glass tubes of the same grade, thickness, and other dimensions were placed in a rack, and to each was added 0.5 to 2.0 cc. of the serum to be mixed with the virus. Just prior to beginning a test at a given temperature, a volume of virus suspension equal to that of the serum was mixed with the serum in each of the required number of tubes, one of which contained a thermometer. One tube of the virus-serum mixture was set aside at room temperature or in the refrigerator as a control. Two or more tubes of the mixture, including one containing the thermometer, were placed in a circulating water bath with a thermostatically controlled electric heating unit set to maintain a temperature of 55, 58, or 60 C. in the bath. As soon as the thermometer in the check tube reached a level just 1 C. below that registered by the thermometer in the water bath, timing was begun. The temperature in the check tube reached the full height of that in the water bath within a few minutes after the tubes were placed in the bath, which never varied more than 0.2 C. above or below the level set for the test. When the end of the prescribed heating period (thirty minutes) was reached, the tubes were immediately removed, and plunged into a bath of iced water, together with the control tube. The tube which had held the thermometer was discarded. The contents of the other heated tube or tubes and the unheated control tube were each inoculated immediately by tunneling and pricking the skin of the metatarsal pads of 3 guinea pigs, the control mixture being inoculated last. Each test, except one, was begun at 55 C., other tubes of the same virus-serum combination being tested at 58 and 60 C. In the test excepted, virus was added to serum from a H.C.-immune hog, a normal bovine serum, and a normal equine

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serum, each of which was tested at 55 and 58 C. only. In all but one of the four complete tests, a control virus-serum mixture, unheated, was set up at the time each virus-serum sample was prepared prior to heating, whether at 55, 58, or 60 C. In the test excepted, only one unheated virus-serum mixture was provided as a control, this being inoculated after all injections of heated mixtures had been completed.

The inoculated guinea pigs were observed once or twice daily for the detection of lesions which sometimes appear as early as twenty-six hours after inoculation and are practically always well developed at forty-eight hours, except when dilute or weak virus is used.

RESULTS OF INFECTIONS WITH HEATED SERUM-VIRUS MIXTURES

Of the 45 guinea pigs, divided into 15 groups, which were injected in the four tests with mixtures of V.S. virus and porcine, bovine, or equine serum, heated at 55, 58, or 60 C. for thirty minutes, only 1 animal (inoculated with material heated 55 C.) developed lesions of V.S. In this case, the lesions were confined to only one of the two inoculated feet and were only about half as well marked as lesions in the animals injected with control mixtures. Only 1 of the 10 control groups, each containing 3 guinea pigs, failed to develop satisfactory lesions of V.S. within forty-eight hours after inoculation. This is the instance mentioned earlier in this paper, wherein half of one unground pad was placed directly in the control serum and the other half was placed in the serum to be heated. These preparations were thoroughly ground in mortars after the one tube had been heated at 55 C. for thirty minutes and, as in the case of all other preparations, were inoculated within thirty minutes after completion of heating and chilling. In this test, 2 of the 3 control guinea pigs, as well as all of those injected with the heated virus-serum mixture, failed to develop lesions. Guinea pigs inoculated with similarly prepared, unheated mixtures, as controls for animals injected with like preparations heated at 58 and 60 C., all developed typical lesions whereas neither group injected with the heated materials did so. It appeared that the pad used for this test at the 55 C. level, at least that portion of it which entered into the preparation of the control tube, contained insufficient virus to produce the disease regularly. In subsequent tests, therefore, a

TABLE I—Results of Tests on Longevity of Infectivity of V.S. Virus Mixed with H.C. Virus after Various Methods of Treatment and Storage in Refrigerator or Incubator

TEST	PREPARATION	RESULTS OF INOCULATIONS OF GUINEA PIGS[4]										
		2d[5]	3d	5d	8d[6]	11d	17d	20d	23d	26d	32d	40d	52d	54d
I-a	C.V.[1], incubated	0/3	0/3	0/3	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
b	C.V., refrigerated	3/3	2/3	2/3	1/3		0/3		0/3		.....	.....	.....	.....
c	Phenol[2], incubated	0/3	0/3	0/3	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
d	Phenol, refrigerated	3/3	3/3	3/3	1/3		1/3		0/3		0/3	.....	.....	.....
e	Untreated, incubated	2/3	2/3	1/3	0/3	.....	.....	.....	.....	.....	.....	.....	.....	.....
f	Untreated, refrigerated	3/3	3/3	3/3	3/3		3/3		3/3		3/3	3/3	0/3	0/3
II-a	Phenol, refrigerated	3/3	3/3	2/3	2/3		1/3		0/3		0/3	.....	.....	.....
b	C.V.[3], incubated	2/3	0/3	0/3	0/3	.....	.....	.....	.....	.....	.....	.....	.....	.....
c	Untreated, incubated	3/3	1/3	0/3	0/3	.....	.....	.....	.....	.....	.....	.....	.....	.....
III-a	Phenol, refrigerated	3/3	1/3	1/3	1/3		0/3		0/3		.....	.....	.....	.....
b	Phenol, refrigerated	.....	.....	1/3	1/3		0/3		0/3		.....	.....	.....	.....

[1]C.V., crystal-violet (phosphate formula)[4]—treated mixture of 8 parts H.C. virus and 1 part V.S. virus.
[2]Phenol (0.5%).
[3]C.V., crystal-violet (glycerin formula)[5]—[6]treated mixture of 8 parts H.C. virus and 1 part V.S. virus.
[4]2d = 2 days' storage, 3d = 3 days' storage, etc.
[5]0/3 = None of 3 guinea pigs inoculated developed lesions of V.S. (Numerator = number of guinea pigs developing lesions; denominator = number of guinea pigs inoculated).
[6]No inoculations made at this interval.

composite suspension of six or more pads from 3 or more guinea pigs, ground in a mortar with saline, was used in each test and no skips were again encountered in the control groups.

EFFECTS OF REFRIGERATION, INCUBATION, PHENOLIZATION, AND ADDITION OF CRYSTAL VIOLET ON V.S. VIRUS

In these tests, three saline suspensions of pads from V.S.-infected guinea pigs, portions of which were used in some of the previously described heating tests, comprised the V.S. virus. This was added to the extent of 1 part to 8 parts of H.C. virus, of which two samples were used, one obtained from a commercial veterinary biological establishment, the other from a pig infected by inoculation of known virus at the Bureau's Animal Disease Station. One mixture of the two viruses, in a test identified as test I, was divided into six portions. Two portions were treated with crystal-violet as in the preparation of crystal-violet H.C. vaccine[4]; two portions were treated with phenol to the extent of 0.5 per cent; two portions were left without treatment. One of each pair of the untreated, phenolized, and crystal-violet treated samples was placed in the incubator at 37.5 C. and the other was placed in the refrigerator at 3 to 5 C. From time to time, samples were taken from each preparation, after thorough shaking, for inoculation into the plantar pads of 3 guinea pigs, the object being to determine how long the V.S. virus remained infective to the extent of producing clinically typical lesions of the disease in the inoculated feet.

The same procedure of inoculation was followed in two additional tests, II and III. In test II, the mixture of H.C. and V.S. viruses was divided into three portions; one of these was phenolized, praparatory to storage in the refrigerator; one was treated with crystal violet[5, 6] prior to incubation; and the last remained untreated, to be held in the incubator.

In test III, two identical phenolized samples of H.C. virus contaminated with V.S. virus were held in the refrigerator for tests at intervals.

In these tests, guinea pigs were inoculated immediately after completion of the various preparations and at intervals of one to twelve days, as long as the samples remained infective. Thereafter, one or more additional inoculations were made as a further check of the noninfectivity of the preparation in question. All guinea pigs injected with the various preparations immediately after they were compounded developed typical lesions of V.S. The results of subsequent tests are summarized in table 1.

These tests show that crystal violet [4, 5, 6] rapidly destroys the infectivity of V.S. virus that is added to H.C. virus as a contaminant. Even when the treated mixture was refrigerated (test I-b), infective virus survivied less than seventeen days and, at the eighth day, it was measurably attenuated. In the incubator, as would be the case in processing crystal-violet H.C. vaccine, crystal violet was still more rapidly virucidal, since neither of two preparations so handled produced lesions after two days (tests I-a and II-b). Untreated mixtures of the viruses still produced V.S. lesions in a third of the inoculated animals after three and five days in the incubator, but on the fifth and eighth days, respectively, the preparations were noninfective. A refrigerated, untreated mixture of the viruses (I-f) produced lesions when inoculated on the fortieth day, but failed to do so on the fifty-second and fifty-fourth days. In the presence of phenol in the amount (0.5%) that is added to simultaneous H.C. viruses, active V.S. virus persisted to the eleventh, seventeenth, and twenty-third days in the refrigerator, in four tests (II-a, I-d, and III-a and b). Finally, one sample of phenolized, incubated mixture of H. C. and V.S. viruses (I-c) failed to produce lesions of the latter when inoculated on the second or subsequent days after preparation.

In consideration of the possibility that V.S. virus capable of infecting swine might still be present in preparations such as those previously mentioned, that were no longer demonstrably infective for guinea pigs, preparations I-b, I-c, and II-b were

injected into three 60-lb. pigs. Five cc. of the three preparations under question were each injected intravenously into a pig and at the same time 35 to 40 cc. of commercial anti-H.C. serum were injected subcutaneously into each pig to protect against any H.C. virus which might have survived. Temperatures of the pigs were taken twice daily, and the animals were regularly observed for approximately one month. No indications of either V.S. or H.C. were observed during this time.

SECONDARY LESIONS IN GUINEA PIGS

In the course of the previously described inoculations of guinea pigs, 198 animals which developed definite V.S. "takes" on the hind feet were observed daily except Sundays for ten to twelve-day periods after inoculation. Sixteen guinea pigs (about 8%) developed secondary lesions on the pads of one or both front feet, three to seven days after appearance of the primary lesions. Lingual lesions, suggestive of V.S., were observed in only one instance and this proved not to be that disease upon subinoculation. It should be mentioned, however, that complete examinations of the oral cavity can not be carried out without anesthesia and this was done on too few occasions to obtain definite data on the occurrence of secondary lesions in this location. Of 67 guinea pigs from which the hind pads were removed for harvest of virus, 4 (approximately 6%) developed secondary lesions. Among 131 guinea pigs on which the primary lesions were undisturbed, 12 (approximately 9%) developed secondary lesions. Thus, there may be a slightly greater tendency toward formation of secondary lesions if the pads showing primary lesions are not removed. The chief interest in the number of infected guinea pigs which develop secondary lesions lies in the relatively few animals that usually so react following inoculation with V.S. virus in contrast with the reported general occurrence of such lesions in experimental foot-and-mouth disease. It should be noted, however, that some strains of V.S. virus do not fail in so large a percentage of guinea pigs to develop "secondaries."[2, 7, 8]

In the course of the guinea pig inoculations here reported, two bacterial organisms were encountered, one a Pasteurella, the other a Salmonella, which caused some deaths in guinea pigs owing to secondary infections such as arthritis and septicemia. It was found that such organisms could frequently be avoided by utilizing tissue from secondary lesions, in preference to that derived from the primary. Otherwise, to obviate troublesome bacterial contaminants, it is desirable if not necessary to return to an earlier uncontaminated source of virus or to resort to filtration.

SUMMARY AND CONCLUSIONS

Vesicular stomatitis (V.S.) virus derived from an outbreak of the disease in swine was repeatedly rendered noninfective for guinea pigs by heating in porcine, equine, or bovine serum at 55, 58, or 60 C. for thirty minutes, although in one of six tests at 55 C., virus of sufficient virulence to infect 1 to 3 guinea pigs survived. All other preparations at 55, 58, and 60 C. were noninfective.

Incubation at 37.5 C. of V.S. virus, added as a contaminant to defibrinated hog-cholera (H.C.) blood rendered the former virus non-infective in five to eight days.

A nonpreserved mixture of V.S. virus and defibrinated H.C. blood produced V.S. lesions in inoculated guinea pigs as late as the fortieth day of storage in the refrigerator but not when tested on the fifty-second and fifty-fourth days.

Three refrigerated, phenolized (0.5%) mixtures of V.S. virus and defibrinated H. C. blood were capable of producing V.S. lesions in inoculated guinea pigs as long as eleven, seventeen, and twenty-three days after preparation.

Crystal violet, as used in the preparation of H.C. vaccine, inactivated V.S. virus added to defibrinated H.C. blood in two to three days.

These tests indicate that properly controlled heating of antiserums at 58 to 59 C. for thirty minutes, as has been required of licensed biological firms by the Bureau, will

render these products free from infective V.S. virus. It is also evident that the virus will not survive the incubation in the presence of crystal violet that is now prescribed for preparing H.C. vaccine (crystal-violet formula). Finally, phenol was found to have only a slowly virucidal action on V.S. virus added to defibrinated H.C. blood held in the refrigerator.

Producers and inspectors of veterinary biological products should be constantly on the alert to detect vesicular diseases, especially in pigs from which it is intended to harvest simultaneous or "hypering" H.C. virus. The close clinical resemblance of vesicular stomatitis, vesicular exanthema, and foot-and-mouth disease necessitates inoculation of several species of test animals for exact differential diagnosis. Biological products prepared on premises where there are or have been animals affected with one of these three virus diseases should be destroyed or so tested as to provide absolute proof of their freedom from active virus.

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Reservoirs of Infection of Streptococcus Agalactiae

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THE PREDOMINANT cause of chronic bovine mastitis, at least in Wisconsin, is *Streptococcus agalactiae*. The habitat of this organism has been found by various investigators to be in the environment of the cow, including the hands of the milkers, in addition to the interior of the udder.

Control of streptococcic mastitis by the sale of infected cows has been successful, but this method is costly and may entail the loss of valuable breeding stock. Segregation of infected cows has given partial success, yet workers have shown that some spread of streptococcic infection occurs in the average dairy herd even though the most accurate and detailed bacteriological methods are used in the diagnosis. The successful control of chronic mastitis attributed to segregation may be due to the sale of infected cows. For instance, in the first year of observation of 4 experimental herds by Plastridge and others,[1] 25 of the 43 infected cows were removed from the premises during the first year; and, even so, 14 cows became newly infected.

In an experimental herd described by Hastings and others,[2] no *Str. agalactiae* were found during two lactation periods; but, when the organism was introduced, it spread to cows milked before as well as after the first infected cow. Since the teat cups were disinfected carefully between milkings, it seems likely that the infection in this herd was spread by means other than direct transfer on the milking machine.

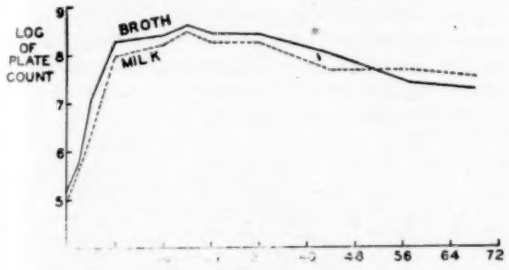
Six dairy herds have been tested regularly for *Str. agalactiae* by the authors, and segregation and sanitary measures have been applied. New infections have appeared in 4 of these 6 herds, though the amount of spread was apparently reduced.

Possible reasons for the failure of segregation to prevent infection with *Str. agalactiae* have been studied. Mattick and others[3] showed that many more infected cows could be detected by enrichment methods of culture than by ordinary plating methods. Thus, with insufficiently sensitive diagnostic methods, cows placed in the uninfected group may be spreading small numbers of the organisms.

Another possible reason for the failure of segregation to prevent the spread of *Str. agalactiae* is the presence of the organisms in places other than in the udder of the cow. Little[4] and Minett[5] believe that *Str. agalactiae* is an obligate organism and that it proliferates only in the udder and its secretions. However, later work indicates that the organism exists in places other than the udder. Bryan[6] reports that he found *Str. agalactiae* in the litter and dust in dairy barns. Lancefield group B streptococci (serologically identical with *Str. agalactiae*) have been recovered from human beings by many workers. Harrison[7] found it on the hands of milkers when they had not been in contact with infected cows for periods varying up to ten days. He believes that the organism became a part of the resident flora of milkers' hands. In a study of 188 strains of group B streptococci, of which 138 were from human sources, Brown[8] states that the human carrier should be considered in attempts to eradicate mastitis from cows.

The length of time that mastitis streptococci can survive outside the udder has been studied by several workers. Klingmuller[9] found that when large numbers of mastitis streptococci were added to test tubes containing straw and bedding, 40 per cent of the tubes contained viable organisms after three and one half months. However, some of his strains of mastitis streptococci had been isolated previously from straw, and the cultural characters he obtained did

not serve to identify the organisms completely. He may have been working with some strains of saprophytic streptococci. Bryan[10] found that alpha, beta, and gamma streptococci survived sixty-six days in sterile sand and nine days in sterile soil, when the inoculum was large. Watts[11] reported prolonged periods of survival ranging up to months outside the animal body, but did not describe his experimental methods. Christiansen[12] reported attempts to infect cows by contamination of the surroundings. He said that Seeleman was able to infect one quarter of a cow exposed to bedding sprayed with a streptococcus. In a similar experiment, Christiansen infected an udder in one quarter of 1 out of 4 cows exposed for thirty-five days to a virulent streptococcus (not *Str. agalactiae*).



Graph 1—Growth curve of Streptococcus agalactiae in milk and broth at 37.5 C.

From all the work described previously, it seems likely that *Str. agalactiae* is an obligate organism. However, conclusive proof of this has not yet been presented. The failure of some to find the organism in the environment of the cow may be due to deficient technique. Furthermore, if *Str. agalactiae* does begin to die as soon as it leaves the udder, the rate of death should be determined. Survival of large numbers of the organism for months in bedding would alter the methods of control necessary to prevent indirect spread of infection. More knowledge on the reservoirs of infection and the means of spread of *Str. agalactiae* seemed (to the authors of this paper) to be necessary for the formulation of an improved control program.

EXPERIMENTAL

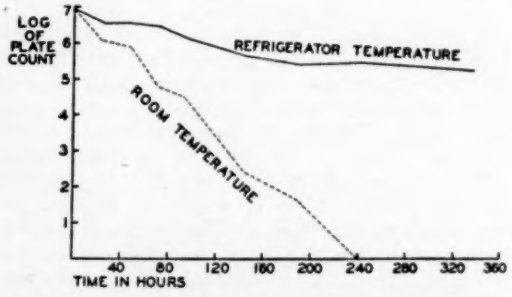
Development and Assay of a Selective Medium.—A medium was developed which would inhibit most organisms in barn dust and barn bedding, but which would permit growth of streptococci. The base medium had the following composition:

Tap water infusion from 4/5 lb. ground veal	920 cc.
Bacto tryptone	10 Gm.
Sodium chloride	5 Gm.
Mannitol	10 Gm.
Agar	15 Gm.

The sterile base medium was melted, cooled to 45 C., and the following added:

Sterile defibrinated bovine blood	50 cc.
Sterile 0.5 per cent solution of brom cresol purple	10 cc.
Sterile 0.5 per cent solution of sodium azide	10 cc.
Sterile 0.02 per cent solution of crystal violet	10 cc.

This medium was suitable for surface and deep plating of *Str. agalactiae* and did not support growth of *Escherichia coli, Aerobacter aerogenes, Bacillus graveolens* or *Staphylococcus aureus.*



Graph 2—Viability of Streptococcus agalactiae in sterile bedding held at room temperature (20-25 C.) and at refrigerator temperature (5-7 C.).

Experiments were performed in which *Str. agalactiae* were added in varying numbers to barn bedding and bovine feces to determine the minimum numbers which could be recovered from the heavily contaminated materials by dilution plating on the selective medium. *Str. agalactiae* could be recovered when as few as 500 were present per gram of bedding. Recovery from feces was more difficult because of the large numbers of other streptococci present. About 100,000 *Str. agalactiae* per gram were necessary for consistent recovery of the organism.

The Identification of Str. agalactiae.—The

pure cultures used for this work had been isolated from cases of bovine mastitis, and in all cases conformed to the criteria of Sherman[13] for the identification of *Str. agalactiae*. The cultures isolated during the work from milkers' hands or other sources were also tested and found to conform to these characteristics.

The Search for Str. agalactiae in the Environment of Infected Cows.—The methods for isolation of *Str. agalactiae* which had been developed and measured in the previous work were now applied in the attempt to find these organisms in the environment of infected cows.

Samples of bedding from barns were collected in sterile bottles. The samples were taken from beneath cows which had one or more quarters infected with the organisms. These samples were immediately plated out on the inhibitor agar medium as follows: Samples were weighed and diluted with 10 parts of sterile tap water. This was mixed by shaking, and 1 cc. of the suspension was put in a 99 cc. blank. The rest of the plating process and identification of colonies were carried out as described previously for bedding to which the mastitis streptococci had been added.

In four such trials made at different times no *Str. agalactiae* were recovered.

Since Bryan[3] had reported success in culturing barn floors for *Str. agalactiae*, his methods were followed as nearly as possible in our attempt to repeat his work. Sterile cotton swabs were moistened in sterile 0.85 per cent saline solution or 2.5 per cent sodium bicarbonate solution, and were rubbed on the floor of the stalls of cows infected with *Str. agalactiae*. The swabs were returned to the solutions. They were streaked on the surface of blood-agar plates containing 1 to 200,000 crystal violet, and were plated in dilution on our inhibitor medium. Colonies were picked and examined, but no *Str. agalactiae* were recovered. Thus, the results of Bryan were not confirmed.

Search for Str. agalactiae on Milkers' Hands.—Harrison[10] found *Str. agalactiae* on the hands of English milkers a long time after the milking period and proved that the organisms became a part of the resident flora of the hands. This indicates that the environmental factor might be significant in the spread of bovine mastitis. His methods were followed to see if the same process occurred in this vicinity. Harrison's report dealt with hand milkers only, and they were said to have washed and disinfected their hands between cows.

In this work, unfortunately, only 2 hand milkers were available. In addition, the hands of 8 persons doing machine milking were tested, and these were selected from operators milking 4 herds having a range from a very low to a very high percentage of infection with *Str. agalactiae*. All were tested from six to eight hours after the last milking.

In testing the hands for the presence of *Str. agalactiae*, a rigorous technique had to be followed to attain success. The hands were first washed thoroughly with soap and water and a stiff bristle brush for three to five minutes. Then they were rinsed for three to five minutes in a solution containing about 200 parts per million of available chlorine. After being dried with a sterile paper towel, the hands were rinsed and brushed thoroughly with 200 or 300 cc. of sterile skimmed milk in a sterile basin using a sterile brush. The milk was then poured into a sterile bottle.

One part of the milk was incubated sixteen to twenty hours and the sediment plated on the inhibitor agar, and one part was plated after standing sixteen to twenty hours in the refrigerator at 5 to 7 C. The rest of the procedure in looking for organisms was carried out as usual.

The hands of 10 milkers in 5 herds were cultured for the presence of *Str. agalactiae*. The organism was recovered from the hands of 2 men who were hand milkers of an extensively infected herd. It was not recovered from 8 men who used milking machines in infected herds and who handled the udders only during the washing and stripping processes.

The Viability of Str. agalactiae in Bedding.—Although *Str. agalactiae* could not be found in bedding collected from the stalls of infected cows, a possibility existed that the organism might be present in numbers too small to be detected. Therefore, information was sought on the rate of death of *Str. agalactiae* when added to bedding under controlled conditions.

Moderately dry bedding was collected from beneath several cows. It was mixed as thoroughly as possible and weighed into sterile vials, usually in amounts of 1 Gm. In some of the trials, the vials were plugged with cotton; in others, screw caps were used to reduce evaporation of moisture. In some of the experiments, the bedding was sterilized by autoclaving at fifteen pounds steam pressure for four hours. Then definite numbers of *Str. agalactiae*, determined by direct counts, were added to each vial of bedding.

The culture used to make the inoculum was transferred in a veal infusion broth medium or in milk three times at 24-hour intervals. The last 24-hour culture was centrifuged to concentrate the organisms. The inoculum was added in as small a volume of fluid as possible, and this varied between 0.1 and 0.5 cc. per gram of bedding; it was mixed as thoroughly as possible.

The culture added to the bedding was in the maximum stationary phase of growth, as can be seen from graph 1 showing the population

curves for pure cultures of *Str. agalactiae* growing in broth and in milk. The data for graph 1 were obtained as follows: A third 24-hour subculture of strain 13c in veal infusion broth medium or milk was used as the inoculum for bottles containing 100 cc. of the same medium in which the inoculum had grown. The milk medium contained brom cresol purple in a concentration of 1 to 20,000. The inoculums for the bottles were estimated to be approximately the same by microscopic counts on stained smears. After inoculation, the bottles were held in a water bath kept at a constant temperature of 37.5 C. Plate counts, and microscopic counts as a check, were made after zero, two, four, eight, sixteen, twenty, twenty-four, thirty-two, forty-four, fifty-seven, and sixty-eight hours of incubation. The same bottle was used to make the counts for the series, and each time before 1 cc. was removed to make a count the bottle was shaken five times. The counts, therefore, are not absolutely accurate because of the breakup of chains by the shakings when taking samples and making dilutions. Taking this error into account, one can still conclude from the curves that growth proceeds at the same rate in broth and in milk and that both cultures are in the maximum stationary phase by twenty-four hours.

The vials of inoculated bedding were held at room temperature, 20 to 25 C., and on one trial a duplicate set was kept in the refrigerator at 5 to 7 C. At about daily intervals one of the vials in the series was removed and cultured by dilution plating. In the experiments in which nonsterile bedding was inoculated, the inhibitor agar medium was used for plating, while for the inoculated sterile bedding the base blood agar was used. Likely colonies on the inhibitor agar were picked into litmus milk for subsequent purification and identification. Counts were made on the plates of the inoculated sterile bedding and, to be sure that the colonies were those of *Str. agalactiae*, one of them was picked in each case and was run through the identification procedure. Graph 2 indicates the rate of death in bedding as affected by temperature.

The results of many other trials similar to the one reported in graph 2 indicate that death of *Str. agalactiae* in bedding at room temperature is fairly rapid. Most of the organisms were dead within twenty-four hours, but a few were found alive for as long as six to nine days. At refrigerator temperatures, the rate of death is much slower, but most of the organisms were dead within a week.

DISCUSSION

The findings reported here indicate certain ways in which *Str. agalactiae* can spread. However, the exact amount of spread which does occur by these means is still to be determined.

Undoubtedly, the spread of *Str. agalactiae* from one herd to another is largely caused by transfer of cows from farm to farm. However, spread within the herd can, and probably does, occur by transfer on the hands of hand milkers. The fact that *Str. agalactiae* can become a part of the resident flora of the hands, as found by Harrison and confirmed in this paper, is important; such knowledge should be considered by both veterinarians and dairy husbandmen. The preventable spread of mastitis streptococci within a herd by the hands of milkers and by the teat cups of milking machines certainly occurs in many cases. But in well managed herds milked by hand the spread of *Str. agalactiae* would not be prevented by ordinary sanitary methods once the organism became established as a part of the resident flora on the milkers' hands. Also, if the organism lives as a resident in or on the skin of the teats, spread may occur *via* the streak canal into the teat canal at any time.

Spread by infected bedding seems less probable, though the possibility cannot be eliminated entirely. The rapid death of the organism at 20 to 25 C. would soon reduce even a heavy contamination of the floor or bedding to insignificant numbers. However, in the so-called "leaker" cows, transfer of infection from one quarter to another on the same cow could readily occur. The fact that cows are usually stabled in the same stall and remain in that stall permanentiy reduces the chance of spread to other cows even though the bedding and floor contain large numbers of organisms. The slow death of *Str. agalactiae* at 5 to 7 C. may allow some indirect spread from infected floors and bedding. In northern areas, such as Wisconsin, many dairy barns remain at near freezing temperatures for several winter months. During this period, indirect spread by transfer on the shoes of the milker would be a distinct possibility.

For a practical application of the results of this work several recommendations could be made:

1) In hand milked herds, the milkers'

hands should be scrubbed thoroughly before the milking begins and should be rinsed in a mild solution of chlorine (about 200 parts per million) between cows. Even this precaution will not prevent transfer if *Str. agalactiae* has become established on the hands, because it may persist for some time.

2) Milkers should avoid getting milk on their hands.

3) Discarded secretions from infected quarters should be milked into a separate pail and not milked onto the floor.

SUMMARY

Streptococcus agalactiae could not be found on the floor or in the bedding from beneath infected cows. It was found as a part of the resident flora of 2 milkers' hands in a hand-milked herd, but could not be found on the hands of 8 dairy attendants in machine-milked herds. *Str. agalactiae* was found to die rapidly in barn bedding; most of the organisms were dead after twenty-four hours, but a few lived as long as six to nine days.

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The Carbon Dioxide Content of the Blood of Dairy Cattle

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THE DAIRY COW is an excellent subject for many blood-chemistry studies. This animal is docile; the blood volume is large, and the drawing of samples of sufficient size for study is not apt to result in blood changes which occur as a result of hemorrhage.[1] Cows are usually maintained on a standardized and well-balanced diet. The daily routine to which they are subjected is well defined. Some cattle, however, are more subject to certain environmental changes than the usual laboratory animals. Those herds which are not housed during the winter are subject to rather wide fluctuations in temperature and variations in weather conditions. That such environmental changes may cause changes in the blood picture due to altered metabolism is logical. Some such changes occur as a result of environmental conditions.[2] Therefore, in any study of the blood chemistry of dairy cows, the effect of environment should first be determined.

More work on the blood chemistry of dairy cattle is justified, not alone because of the clinical value which it may have for the veterinarian but also for the general theoretical implications of such work.

The experiments presented in this paper represent an effort to: (1) establish the normal carbon dioxide content of the blood of healthy dairy cows at various external temperatures, (2) compare the carbon dioxide content of blood from two different veins, (3) determine the effect of the estrous cycle on the blood-carbon dioxide content, and (4) compare the carbon dioxide content of blood from cattle with brucellosis with that from normal cattle. A total of 105 experiments on 42 animals is included in this report.

TABLE I—The Normal Carbon Dioxide Content of the Blood of Healthy Dairy Cows at Various External Temperatures

TEMP.	HOLSTEIN-FRIESIAN				JERSEY			
	176	205	194	234	29	121	34	93
27	....	....	....	69.5	62.1	....	....	....
28	....	....	....	56.2-58.5	57.2-55.8	....	....	....
30	....	....	62.5	....	....	....	62.5	....
31	....	....	64.2	....	....	....	64.2	....
32	....	....	59.5	64.2	54.6	....	57.5	....
35	....	....	56.0-62.2	....	....	....	58.6-60.8	....
37	....	....	....	....	57.0	....	....	....
39	....	....	54.5	....	....	....	57.2	....
43	....	....	54.2	....	....	....	52.5	....
45	64.4-54.7	63.0-51.7	....	....	....	56.2-48.7	....	50.7-50.7
46	49.9	52.2	....	....	....	46.7	....	48.4
49	....	....	53.5	....	....	....	51.7	....
50	52.5	49.2	....	....	....	50.2	....	50.2
53	....	....	58.8-62.4	....	....	....	49.5-50.3	....
58	49.2	48.7	....	....	....	47.5	....	46.7
60	....	....	58.6	....	....	....	58.8	....
61	52.7	52.5	....	....	....	49.5	....	48.5
62	55.7	56.2	....	....	....	43.5	....	51.8
63	57.2	57.1	....	....	....	48.0	....	49.7
67	58.6	60.1	....	....	....	50.0	....	50.7
70	57.8-61.4	53.0-51.5	....	....	....	50.4-57.5	....	50.2-50.4
73	54.9	56.8	....	....	....	46.4	....	49.2
81	61.7	52.0	....	....	....	46.5	....	50.7

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We wish to express our appreciation to Mr. M. B. Harrison for assistance in the mathematical determination of the curves contained herein.

EXPERIMENTAL

The normal animals used in these experiments were from the college Jersey and Holstein-Friesian dairy herds. All of these animals had been negative to the standard plate agglutination test for brucellosis throughout the past two years. Eight Jersey cows from a neighboring dairy were used as well as 2 Holstein-Friesian nurse cows and 2 Aberdeen-Angus cows from the college beef cattle herd.

The animals were brought into the veterinary hospital, placed in stocks, and the blood samples obtained. About one half of the samples were drawn at 9 a.m. The remainder were obtained at 1:30 p.m. The samples taken in the morning were during the colder months of the year, and the cattle were not housed during the night. Blood was drawn two successive times from the same vein of each cow before changing to the opposite side. No phlebitis was produced in any of these animals in spite of the fact that some were bled as many as 13 times. The samples were drawn by gravity into oiled syringes from the subcutaneous abdominal vein or the jugular vein, according to the conditions of the experiment. They were expelled immediately under oil into oxalated tubes which were then stoppered in such a manner that they were completely sealed from the air. They were analyzed without delay, duplicate determinations of the carbon dioxide content of the whole blood being made according to the volumetric method of Van Slyke.[3] No more than four samples were ever drawn at one time. One determination of the carbon dioxide content of each sample was made. The determinations were then repeated in order. This procedure was followed to determine if the slight amount of necessary standing of samples was accompanied by any change in the carbon dioxide content. No such change was detected at any time.

DISCUSSION

1) *The Effect of Temperature on Blood–Carbon Dioxide Content.*—Figure 1 was obtained by determining the regression curve by applying the method of least squares to the experimental values for the blood-carbon dioxide content of the Jersey cows at various temperatures. There is a decrease in the carbon dioxide content as the temperature increases, as indicated by the slope of the curve obtained. Examination of table 1 will reveal that this decrease is only a general tendency, since occasional values for the carbon dioxide content of a given animal are completely out of line with the temperature effect. This can only mean

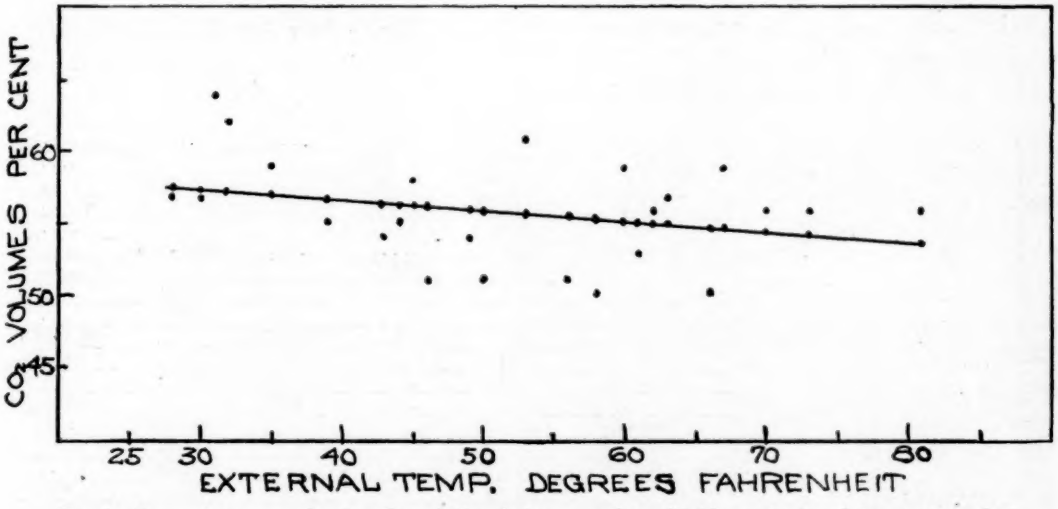

Fig. 1—Regression curve showing the relation between carbon dioxide content and temperature for the Jersey cows.

that other factors, some perhaps environmental, others metabolic, have a profound influence on the carbon dioxide content of the blood. These factors can, on occasion, completely overshadow the effect of temperature.

That the Holstein-Friesian animals are not influenced to the same extent by changes in temperature is evident from the lack of slope in the curve shown in figure 2. This curve was obtained in the same way as that for the Jerseys. It is not exactly clear why one breed of animal should be more affected by temperature changes than another. Nevertheless, we believe this to be a real difference. The average blood-carbon dioxide of the Jerseys at temperatures below 50 F. was 56.5 volumes per cent. The average value for temperatures above 50 F. was 50.0 volumes per cent. For the Holstein-Friesian cows the average value for temperatures below 50 F. was 56.9 volumes per cent; above 50 F. the average carbon dioxide content was 55.0 volumes per cent.

The average carbon dioxide content for all the Holstein-Friesians was 56.5 volumes per cent. For the Jerseys, the average was 53.6 volumes per cent. While there is a difference in this respect between these two breeds, it is not as great as the difference between the carbon dioxide capacity of the blood for the same two breeds as reported by Anderson and others.[4]

2) *The Carbon Dioxide Content of the Blood from the Jugular Vein Compared with That from the Subcutaneous Abdominal Vein.*—In experiments on 8 cows, the carbon dioxide content of blood from the jugular vein varied from 47.3 to 62.0, with an average of 52.9 volumes per cent. In the same eight experiments, the blood from the subcutaneous abdominal vein showed a minimum of 44.7 and a maximum of 64.0 volumes per cent. The average in the latter case was 53.2 volumes per cent.

The blood from the jugular vein contained a slightly greater amount of CO_2 in four out of eight cases; however, in only one case is the difference beyond the maximum experimental error which has been estimated to be not more than 2.0 volumes per cent. In one case, the CO_2 content of the blood of the subcutaneous vein was 3.3 volumes per cent higher than that from the jugular vein. It is thus obvious that the carbon dioxide content of the blood from these two veins is not significantly different. This may seem surprising in view of the relatively greater activity of the region drained by the subcutaneous abdominal vein. On the other hand, that may be expected because of the much larger volume

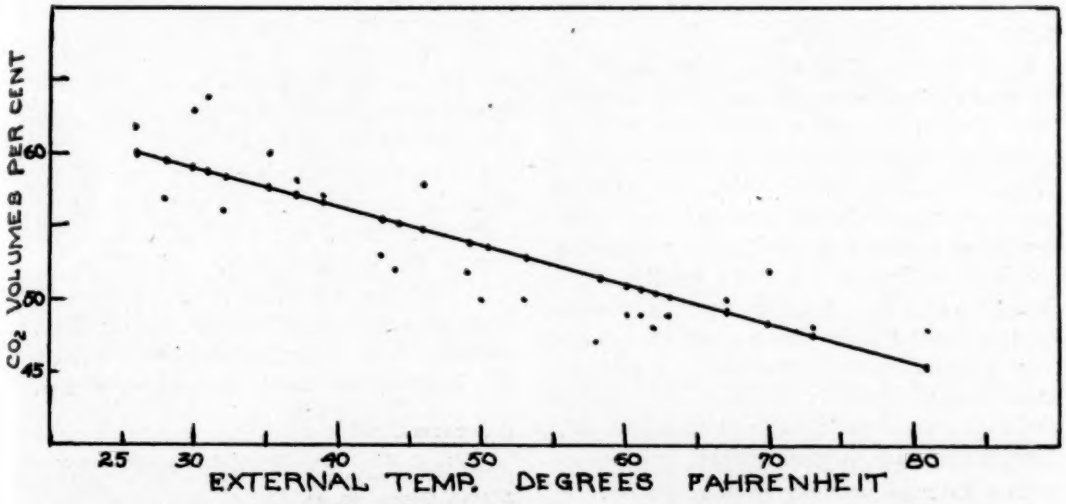

Fig. 2—Regression curve showing the relation between carbon dioxide content and temperature for the Holstein-Friesian cows.

of blood involved in this case. The total quantity of carbon dioxide carried by the blood in these two veins is undoubtedly different.

3) *The Carbon Dioxide Content During the Estrous Cycle.*—In the cases of 2 of the animals studied, we have observed an apparent relation between the carbon dioxide content of the blood and the stage of the estrous cycle. However, there did not appear to be any such general relationship, as is evident from the average carbon dioxide content at different stages in the estrous cycle. The average for the period from one to six days following estrus was 56.3, for seven to twelve days 56.7, for thirteen to eighteen days 57.6, and for nineteen to twenty-one days 56.2 volumes per cent. The 19- to 21-day period includes estrus as well as those days immediately prior to estrus. It is doubtful if the slight rise in carbon dioxide content toward the end of the period (thirteen to eighteen days) is of significance.

4) *The CO_2 Content of the Blood of Normal Cows Compared with That of Cows with Brucellosis.*—The blood from each of the animals used in these experiments was drawn from the jugular vein. The cows in each pair were selected from the same herd, one being positive to the agglutination test for brucellosis and the other negative. All factors which may have affected the carbon dioxide content in these animals should have been about the same with the possible exception of their position in the estrous cycle. It has already been pointed out that this is probably without effect.

The average carbon dioxide content of the blood of the negative cows was 52.0 volumes per cent and that of the positive cows 54.5 volumes per cent. The difference of 2.4 volumes per cent is only slightly greater than the estimated maximum experimental error. In two pairs, there was a significant difference, the positive cow's blood having a higher carbon dioxide content. In one pair, the positive cow's blood had only slightly more carbon dioxide and in the remaining pair, the negative animal had slightly more.

A higher carbon dioxide tension in the tissues of animals susceptible to, or infected with, brucellosis might be favorable to the growth of the organism. Such a higher carbon dioxide tension should be reflected in the carbon dioxide content of the blood. Further investigation of this problem is desirable.

CONCLUSIONS

1) The average blood-carbon dioxide content for Jersey cows was determined to be 53.6 volumes per cent. This was shown to vary inversely with temperature.

2) The average blood-carbon dioxide content of Holstein-Friesian cows is 56.5 volumes per cent. The variation with changes in temperature is not so pronounced as in the case of the Jerseys.

3) The carbon dioxide content of the blood from the jugular vein does not differ significantly from that from the subcutaneous abdominal vein.

4) There appears to be no general relationship between the carbon dioxide content of the blood of an animal and its position in the estrous cycle.

5) In two experiments, cows with brucellosis had a higher average carbon dioxide content than noninfected cows; however, this has not been true in all experiments.

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The Etiology of Calf Diphtheria

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ACTINOMYCES NECROPHORUS has long been considered the causative agent of calf diphtheria, but the etiology of the disease has received little experimental attention. Scrivner and Lee[1] found that ground material from an active case would produce a resemblance to the original lesion when the material was introduced into the oral tissues of calves by injection or scarification, but pure cultures of *A. necrophorus* isolated from naturally occurring cases failed to produce definite lesions in calves, and various products of the organism failed to reveal any prophylactic value in limited field trials. They indicated that the rôle of secondary invaders might be an important one. Farquharson[2,3] has suggested that possibly other agents in addition to *A. necrophorus* should be considered as causes, but he includes no experimental evidence to substantiate this. In another disease long attributed to *A. necrophorus*, the venereal form of lip and leg ulceration, Tunnicliff and Matischeck[4] have incriminated a virus.

While calf diphtheria often responds readily to treatment with the sulfa drugs, many cases are lost because either treatment is omitted or is administered too late. Since the disease occasionally assumes serious proportions on certain premises, prevention would be preferable to treatment. Allred, Brown, and Murie have described[5] the ravages of necrotic stomatitis in a herd of Wyoming elk held on winter feed grounds. Any large scale handling of elk would be impractical, but an effective means of vaccination might prove useful. Aside from theoretical considerations, an exact knowledge of the etiology of calf diphtheria would be necessary in any attempt to develop an effective means of immunization.

Approved for publication by J. A. Hill, director of the Wyoming Agricultural Experiment Station, Laramie.

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PROCEDURE

As it was impractical to secure experimental calves at the time, the possibility of a virus being the primary cause was not investigated because of the likelihood of host specificity. Instead, the rôle of secondary invaders was studied by employing them in conjunction with *A. necrophorus* in rabbits. Material was secured from three fatal cases of calf diphtheria on different ranches, and all possible bacterial species were recovered directly from the lesions and, to a certain extent, from related structures, as the lymph nodes draining the area. Specimen 1 consisted of necrotic tissues from the larynx; *A. necrophorus* could be demonstrated microscopically but was not recovered. Specimens 2 and 3 were calf heads received in good condition. In both instances the necrotic lesions were confined to the tongue and mouth; *A. necrophorus* was recovered in each case. Efforts to avoid overlooking any species resulted in a duplication of some isolations. Table 1 shows the miscellaneous flora encountered and, to a certain extent, is indicative of their relative abundance. In addition, two strains of streptococci of the cultures secured from a fatal case of necrobacillosis, confined to the mouth of a rabbit, were included on the possibility that later employment in the same species might provoke more positive results than if isolated from calves.

The general procedure was to inject small amounts of these ancillary cultures and *A. necrophorus*, intradermally, into the upper buccal surface of the lips of rabbits. As the primary concern was an evaluation of these ancillary cultures, one strain of *A. necrophorus*, recovered from specimen 3, was used throughout. It was found that 0.01-cc. and 0.1-cc. amounts of this strain, as originally isolated, invariably failed to produce a definite lesion, although 0.5 cc. did, and when passed through a rabbit, it was capable of doing so in 0.1-cc. amounts. Except for an early experiment in which 0.05 cc. was employed, 0.1 cc. of the original stock strain, incapable of producing a lesion alone, was injected. The ancillary cultures were also employed in 0.1-cc. amounts, except for variations from 0.05 to 0.3 cc. in the early work. After using cultures of different ages, 72-hour cooked meat broth, with

added defibrinated blood, cultures of *A. necrophorus*, and 48-hour plain or 1 per cent tryptose broth cultures of the ancillary organisms were employed routinely. The anaërobic lactobacilli were grown in Hibler's medium. The culture of *A. necrophorus* was maintained by weekly transfers in cooked meat medium with added blood, and at intervals of a month, these cultures were plated out anaërobically on blood-agar plates to insure purity. Cultures were further checked for purity immediately before use by microscopic examination. With the exception of the anaërobes, the ancillary cultures were kept on agar slants stored in the refrigerator, except when transferred at monthly intervals.

Because of the site selected for inoculation, it was difficult in all cases to be certain of the intradermal delivery of the full amount, but every effort was made to keep the inoculation as close to the surface as possible. As necrotic stomatitis is sometimes thought to be associated with injury from awns or teething, in some instances, the rabbits' lips were lightly bruised with pliers before the injections were made. However, this seemed an unnecessary procedure and was omitted from the later experiments.

After the intradermal injections of *A. necrophorus* plus the ancillary culture in one lip and of the ancillary culture alone in the other, the rabbit was examined daily for the first week and again at the end of the second week. In a few cases, *A. necrophorus* and the ancillary culture were introduced into one lip and *A. necrophorus* was used alone in the other lip, or *A. necrophorus* was employed alone in both lips in a control rabbit until the inability of *A. necrophorus* to produce lesions in the amount employed was established.

RESULTS

A. necrophorus was present in the three specimens examined. As indicated in table 1, enterococci, pyogenic streptococci, and micrococci were recovered from two specimens. One of these also yielded staphylococci, corynebacteria, and a yeastlike organism resembling *Endomycoptes albicans*, and the other, a viridans streptococcus. The flora of the remaining case consisted of staphylococci and a corynebacterium. The remainder of the bacteria isolated were not consistently present and were essentially saprophytic in nature.

With the amounts of the *A. necrophorus* strain employed, it was incapable of producing lesions on intradermal injection into the lips of rabbits. Used with a wide variety of other cultures recovered from necrotic lesions, definite lesions were produced in the greater proportion of such experiments. These ancillary cultures, with a few exceptions, were also incapable of pro-

TABLE 1—Summary of Rabbit Inoculations

Source of Material	Organisms isolated	No. of strains isolated	Inoculations Made: In conjunction with *Actinomyces necrophorus* — Total injections	In conjunction with *Actinomyces necrophorus* — No. positive	Organisms Alone — Total injections	Organisms Alone — No. positive
Specimen 1	*Streptococcus liquefaciens*	10	17	13	17	0
	Streptococcus durans	6	11	11	11	0
	Streptococcus sp. (animal pyogenes)	1	1	1	1	0
	Micrococcus cereus	1	2	2	2	0
	Bacillus mesentericus	4	7	5	7	0
	Lactobacillus bifidus	2	2	1	2	0
	Achromobacter liquefaciens	1	2	1	2	0
	Achromobacter formosum	1	2	1	2	0
	Culture lost before identification completed: Gram negative rod not fermenting common carbohydrates.	1	1	0	1	0
Specimen 2	*Corynebacterium flavidum*	1	2	0	2	0
	Staphylococcus aureus	2	7	3	7	3
	Staphylococcus albus	1	1	0	1	0
	Staphylcoccus epidermidis	2	2	1	2	0
Specimen 3	*Streptococcus liquefaciens*	2	2	2	2	0
	Streptococcus salivarius	1	1	1	0	0
	Streptococcus sp. (animal pyogenes)	1	1	1	1	0
	Staphylococcus albus	4	8	5	6	1
	Staphylcoccus epidermidis	2	2	2	1	0
	Corynebacterium	1	1	1	1	1
	Corynebacterium?	2	2	2	1	0
	Micrococcus subgranulatus?	1	1	0	1	0
	Achromobacter butyri?	1	1	1	0	0
	Vibrio aquatilis?	1	1	1	1	0
	Yeastlike	2	10	7	9	1
Isolated from necrobacillosis in rabbit.	*Streptococcus durans*	2	2	2	2	0

TABLE 2—Effect of Vitamin Deficiency

Test lip	Result	Control lip	Result	Remarks
A. necrophorus ..... *Str. durans* (Case 1, culture 9)	3+	*Str. durans* (9)	—	Fed oats only. Vit. A*-22.0.
A. necrophorus ..... *Str. durans* (9)	3+	*Str. durans* (9)	—	Fed oats and hay. Vit. A-66.2.
A. necrophorus ..... *Str. durans* (10)	3+	*Str. durans* (10)	—	Fed oats only. Vit. A-18.9.
A. necrophorus ..... *Str. durans* (10)	1+	*Str. durans* (10)	—	Fed oats and hay. Vit. A-47.6.
A. necrophorus ..... *Str. durans* (11)	4+	*Str. durans* (11)	—	Fed oats only. Vit. A-28.3.
A. necrophorus ..... *Str. durans* (11)	3+	*Str. durans* (11)	—	Fed oats and hay. Vit. A-66.2.
A. necrophorus ..... *B. mesentericus* (12)	4+	*B. mesentericus* (12) ...	—	Fed oats only. Vit. A-22.
A. necrophorus ..... *B. mesentericus* (12)	3+	*B. mesentericus* (12) ...	—	Fed oats and hay. Vit. A-45.7.
A. necrophorus ..... *Str. liquefaciens* (13)	4+	*Str. liquefaciens* (13) ..	—	Fed oats only. Vit. A-31.5.
A. necrophorus ..... *Str. liquefaciens* (13)	2+	*Str. liquefaciens* (13) ..	—	Fed oats and hay. Vit. A-63.
A. necrophorus ..... *Str. durans* (14)	3+	*Str. durans* (14)	—	Fed oats only. Vit. A-25.2.
A. necrophorus ..... *Str. durans* (14)	2+	*Str. durans* (14)	—	Fed oats and hay. Died fourth day.
A. necrophorus ..... *Str. liquefaciens* (15)	3+	*Str. liquefaciens* (15) ..	—	Fed oats only. Vit. A-13.9.
A. necrophorus ..... *Str. liquefaciens* (15)	3+	*Str. liquefaciens* (15) ..	—	Fed oats and hay. Vit. A-62.9.
A. necrophorus ..... *Str. liquefaciens* (16)	4+	*Str. liquefaciens* (16) ..	—	Fed oats only. Vit. A-15.8.
A. necrophorus ..... *Str. liquefaciens* (16)	2+	*Str. liquefaciens* (16) ..	—	Fed oats and hay. Vit. A-47.4.
A. necrophorus ..... *Str. liquefaciens* (5)	4+	*Str. liquefaciens* (5) ...	—	Fed oats only. Vit. A-4.7.
A. necrophorus ..... *Str. liquefaciens* (5)	4+	*Str. liquefaciens* (5) ...	—	Fed oats and hay. Vit. A-69.38.
A. necrophorus ..... *Str. liquefaciens* (6)	4+	*Str. liquefaciens* (6) ...	—	Fed oats only. Died second day.
A. necrophorus ..... *Str. liquefaciens* (6)	2+	*Str. liquefaciens* (6) ...	—	Fed oats and hay. Vit. A-63.0.

*No carotene found. Plasma Vitamin A expressed as mμ per cent.

Cultures injected in 0.1-cc. amounts throughout. Rabbits kept on indicated rations beginning two months prior to experiment. Vitamin assay five to seven days after inoculation. Relative severity of lesions indicated by 4+, 3+, etc.

ducing lesions when used alone, but even those which might be thought of as soil or water forms contributed to the development of lesions with *A. necrophorus* in about half the attempts. One strain of corynebacterium was capable of producing lesions alone; *Staphylococcus aureus* and, to a much lesser extent, *Staphylococcus albus* also possessed this property, as did the yeastlike organism. That *A. necrophorus* was not entirely dependent upon such symbiotic relationship was illustrated when the same culture in similar amount, after animal passage, was capable of producing a lesion comparable to that produced by the combined organisms.

Invariably, if positive results were to follow inoculation, a lesion, usually a small white spot or a larger pale area, developed in twenty-four to forty-eight hours. Two types of lesions were seen. In one, the injury was confined to the surface, with erosion and the development of a necrotic surface very much resembling the lesions observed in calves. In the other, a hard nodule developed in the substance of the lip. The latter frequently protruded into the mouth, when its surface was yellowish. At some stage, pus always was released; if a copious discharge occurred, the duration of the lesion was lessened. With the lesion confined to the surface, healing was apparent and frequently well advanced at the end of two weeks; the deeper seated lesions were frequently more persistent. Some of the rabbits were used repeatedly in the experiment; in a few cases, nodules remained and prevented the use of the rabbit a month

after the termination of the previous experiment. In one case, several months afterwards, fatal necrobacillosis with lesions confined to the head developed. Another rabbit later developed numerous abscesses about the tendon sheaths and joints, a condition described as typical, following the intravenous injection of *A. necrophorus* (Beveridge[6]).

When a small group of recently weaned rabbits was found to be somewhat refractory to these inoculations, it was thought the vitamin intake might have influenced the results. One experiment is given in detail in table 2 to illustrate the results obtained in the presence of marked vitamin deficiency, as well as to indicate the procedure of these inoculations as a whole. While more than one vitamin would be deficient under the conditions present, only evaluation of the levels of vitamin A, associated with the resistance of the mucous membranes to infections, was undertaken. Although the results are not outstanding, consistently greater lesions were found in the vitamin deficient rabbits. Reversing the process and supplying cod liver oil or ascorbic acid, while using adult and recently weaned rabbits as controls on the usual ration, did not produce any conclusive results or substantiate the earlier observation that young rabbits might be more resistant.

If one takes into consideration that these experiments utilized the ancillary bacteria found in a disease of one species in artificial attempts to reproduce it in another in association with an attenuated strain of *A. necrophorus*, one could well conclude that the development of necrotic mouth lesions is markedly increased by the symbiotic relationships between *A. necrophorus* and potentially pathogenic organisms expected to be found in the mouth, and to a lesser degree by saprophytic forms. Some of these bacteria would apparently be capable of initiating the development of lesions without the association of *A. necrophorus*.

CONCLUSION

1) In addition to *Actinomyces necrophorus*, enterococci, pyogenic streptococci, staphylococci, micrococci, and corynebacteria together with saprophytic bacteria were recovered from naturally occurring cases of calf diphtheria.

2) Injecting intradermally a strain of *A. necrophorus* incapable of producing lesions into the buccal surface of rabbits' lips in conjunction with cultures of secondary invaders resulted in abscess formation or surface necrosis. By increasing the amount injected, and after animal passage, the strain of *A. necrophorus* was capable of initiating such lesions alone. All strains of *Staphylococcus aureus* and one strain of *Staphylococcus albus*, of a corynebacterium, and of a yeastlike organism were also capable of producing a lesion without the assistance of *A. necrophorus*. Lesions followed in about half of the inoculations where an *A. necrophorus*-saprophytic culture combination was employed and in most cases where *A. necrophorus* was used with a potential pathogen.

3) Marked vitamin deficiency increased the severity of the lesions observed, but conversely, feeding cod liver oil or ascorbic acid did not decrease the severity under that for control groups.

ACKNOWLEDGMENTS

Our appreciation is expressed to the Wyoming veterinarians who have supplied us with calf diphtheria material and to the Research Chemistry Department, University of Wyoming, for placing their equipment for vitamin assay at our disposal.

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Chemotherapy of Trichomonas Foetus (Protozoa) in Vitro

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AT THE PRESENT time no satisfactory therapeutic agent has been established for the successful treatment of bovine trichomoniasis. *In vitro* studies of the trichomonacidal properties of various chemicals may offer useful information for future *in vivo* applications. The purpose of this paper is to present the *in vitro* effects of 350 compounds on *Trichomonas foetus*. The data presented in this paper were obtained from tests of a number of proprietary compounds which were requested from various manufacturers, as well as others chosen because of their widespread veterinary use.

REVIEW OF LITERATURE

Daust[1] was the first to report *in vitro* studies with therapeutic agents against *T. foetus*. He used a microscopic test in which 2 cc. of the drug were mixed with 5 cc. of uterine fluid containing *T. foetus* and which were examined many times daily for several days. Daust found that 100 per cent Tromsulin, 100 per cent Batticon, 1:1000 entozon solution, 1:1000 to 1:1500 chinosol solution, 2.5-5.0 per cent Neurogen and 5.0 per cent sodium chloride solution killed *T. foetus* within twenty-four hours.

Senna[2] demonstrated the effects of 54 different compounds by mixing 5 cc. of a 48-hour *T. foetus* culture grown in Ubertini's[3] blood broth with 5 cc. of the test solution. Microscopic readings were made at one, three, five, and thirty minutes and one, two, six, and twenty-four hours. After the trichomonads were immobile, 0.5 cc. of the liquid was withdrawn, inoculated into blood broth, and examined two days later for motility and multiplication. His results were as follows:

Killed in One Minute.—0.01 per cent mercuric iodide, 0.05 per cent mercuric chloride, 0.1 per cent malachite green, 0.1 per cent salicylic acid, 0.1 per cent iodine, 0.3 per cent picric acid, 0.5 per cent hydrochloric acid, 0.5 per cent chromic acid, 0.5 per cent cresol, 0.5 per cent acetic acid, 0.5 per cent sodium hydroxide, 0.5 per cent potassium permanganate, 1.0 per cent formaldehyde, 1.0 per cent phenol, 1.0 per cent lysoform, 2.0 per cent sulphuric acid, 2.0 per cent chloroform, 2.0 per cent Tionina, 2.0 per cent silver nitrate, 3.0 per cent methyl violet, 4.0 per cent creolin, 5.0 per cent thymol, 5.0 per cent silver protein (protargol), 5.0 per cent menthol, 5.0 per cent bicarbonate of soda, 5.0 per cent Rivanol, 5.0 per cent calcium chloride, 10.0 per cent calcium hydroxide, 10.0 per cent ether, 10.0 per cent Lugol's solution, 10.0 per cent sodium chloride, 10.0 per cent lactic acid, 15.0 per cent ethyl alcohol, 15.0 per cent acetone, 15.0 per cent water of Salso, and 15.0 per cent glycerine.

Killed in Three Minutes.—10.0 per cent copper sulfate and 20.0 per cent zinc sulfate.

Killed in Five Minutes.—5.0 per cent entozon and 10.0 per cent boric acid.

Killed in Thirty Minutes—50.0 per cent hydrogen peroxide.

Killed in One Hour.—3.0 per cent trypaflavin, 5.0 per cent potassium bichromate, 10.0 per cent atoxyl, 10.0 per cent sodium salt of Stovarsol (acetarsone), and 10.0 per cent tartar emetic.

Killed in Six Hours.—5.0 per cent Devegan and 10.0 per cent calomel.

No Effect.—methylene blue and quinine sulfate.

Mussill[4] tested 56 compounds by adding by volume 0.2 of a drug solution with 0.8 by volume of a 48-hour trichomonad culture grown in Diernhofer's[5] medium. The organisms were examined immediately, at two hours, and at twenty-four hours. Mussill found that the following chemicals would kill *T. foetus* immediately: 0.02 per cent mercuric chloride, 0.02 per cent mercuric oxycyanide, 0.01 per cent silver nitrate, 1.0 per cent copper sulfate, 1.0 per cent copper chloride, 1.0 per cent zinc chloride, 1.0 per cent zinc sulfate, 0.2 per cent sodium fluoride, 0.2 per cent sodium perborate, 4.0 per cent ammonium sulfide, 0.05 per cent chloramine, 20.0 per cent Lugol's solution (1:2:100), 2.0 per cent sodium bisulfite, 0.5 per cent Sagrotan, 4.0 per cent ichthyol, 0.01 per cent ichthargan, 0.5 per cent Zimtaldehyde, 0.05 per cent chinosol, 1.0 per cent saponin, 0.1 per cent trypaflavine, 1.0 per cent soda, 5.0 per cent sodium hydrocarbonate, 1.0 per cent hard soap, 0.5 per cent soft soap, 10.0 per cent alcohol, chloroform. Compounds which were lethal to *T. foetus* within two hours included 0.2 per cent sodium arsenite, 2.0 per cent sodium sulfite, 1.0-0.01 per cent formalde-

(Continued on page 48.)

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From the Department of Veterinary Science, University of Wisconsin, Madison.

TABLE I—Compounds Which Killed Trichomonas Foetus in One Minute

Chemical Name	Per Cent	pH of Test Compound
1. Acetaldehyde	3.0	4.36
2. Acetic acid	0.5	3.20
3. Acetic anhydride	0.3	3.30
4. Acetone (dimethylketone)	18.0	7.80
5. Alcohol, butyl	10.0	7.58
6. Alcohol, butylic iso	6.0	6.83
7. Alcohol, ethyl (grain alcohol)	20.0	5.88
8. Alcohol, methyl (wood alcohol)	25.0	7.68
9. Alcohol, propyl iso	18.0	7.83
10. Alkyldimethylbenzylammonium chlorides (Concentrate Zephiran chloride—aqueous sol. 12.8%)	0.1*	6.46
11. Alkyldimethylbenzylammonium chlorides (Zephiran chloride — tinted tincture 1:1000)	10.0	4.96
12. Alkyldimethylbenzylammonium chlorides (Zephiran —aqueous sol. 1:1000)	12.0	6.35
13. Alkyldimethylbenzylammonium chlorides (Zephiran —stainless tincture 1:1000)	12.0	6.40
14. Aluminum ammonium sulfate (ammonium alum)	16.0	3.23
15. Aluminum chloride	8.0	3.80
16. Aluminum potassium sulfate (alum)	15.0	3.26
17. 2-Amino-4-arsenosophenol hydrochloride (Mapharsen)	12.0	2.39
18. 2-Amino-4-arsenosophenol hydriodide	0.1	2.96
19. β-(2-Amino-4-arsenosophenoxy) ethanol	2.0	5.75
20. β-(2-Amino-4-arsenosophenoxy) propanol-2	2.0	6.56
21. 2-Amino-8-dimethylaminophenothiazinium chloride (Azur A)	2.0	3.13
22. 5-Aminosalicylic acid hydrochloride	2.0	1.72
23. Ammonium acetate	15.0	6.90
24. Ammonium bromide	10.0	5.92
25. Ammonium chloride	6.0	5.35
26. Ammonium thiocyanate	6.0	5.26
27. Anhydro-6-(hydroxymercuri) salicylic acid	Sat.†	8.63
28. Aniline	3.0	8.52
29. Aniline hydrochloride	8.0	2.73
30. Anhydro-2-nitro-4, 6-di(hydroxymercuri) resorcinol	Sat.	8.00
31. Anhydro-2-nitro-3-hydroxymercuri-4-hydroxy-5-methylphenol	Sat.	8.10
32. Barium chloride	10.0	6.95
33. Barium iodide	5.0	11.54
34. 1, 2-Benzenediol (catechol)	2.0	5.90
35. 1, 3-Benzenediol (resorcinol)	3.0	5.80
36. Benzoic acid	0.3	3.30
37. o-Benzylphenol	0.3	6.24
38. Boric acid + benzoic acid + menthol + methyl salicylate + eucalyptol + thymol + alcohol (Listerine)	30.0	4.79
39. Boric acid + benzoic acid + thymol-eucalyptol + menthol + methyl salicylate + oil of thyme + alcohol (Mi 31 sol.)	30.0	3.56
40. Brilliant green	0.1	3.53
41. 5-Bromosalicylic acid	Sat.	2.86
42. Butyric acid	0.5	3.23
43. Cadmium chloride	5.0	5.38
44. Cadmium iodide	3.0	6.85
45. Calcium chloride	9.0	7.50
46. Calcium dimethyldithiocarbamate	4.0	9.50
47. Cetylpyridinium bromide	0.01	6.06
48. Cetylpyridinium chloride (Ceepryn)	0.003	7.80
49. Cetylpyridinium chloride (Ceepryn — aqueous sol. 1:1000)	5.0	7.62
50. Cetyltrimethylammonium bromide	0.01	6.45
51. 3 Chloro-4 hydroxy diphenyl	Sat.	7.40
52. 4-Chlororesorcinol	0.4	5.52
53. 5-Chlorosalicylic acid	Sat.	2.93
54. Chlorothymol + boric acid + malic acid + citric acid + alcohol (Pepsodent Antiseptic)	23.0	6.25
55. Chlorothymol colloidal (Clymocol)	0.07	6.17
56. Chromium trioxide (chromic acid)	0.3	1.95
57. Citric acid	8.0	2.03
58. Citric acid + papain + lactose + methylsalicylate + eucalyptol + menthol + chlorthymol (Meta Cine Douche Powder)	3.0	2.49
59. Cresylic acid	0.3	7.49
60. Cresylic acid + neutral soap + glycerine (Lysol disinfectant)	0.07	7.38
61. Cresylic acid + glycerine (cresylol)	0.05	8.82
62. Cupric sulfate	9.0	3.89
63. Decamethylene bis-(decyldimethylammonium bromide)	0.02	6.86
64. Detergent germicidal and insecticidal (10% active ingredients)	0.02	6.20
65. Detergent liquid germicidal (10% active ingredients)	0.02	6.26
66. 4, 4'-Diamidinodiphenoxypentane (Pentamidine)	2.0	3.65
67. 4, 4'-Diamidinodiphenylether (Phenamidine)	10.0	3.69
68. 3, 6-diaminoacridine dihydrochloride (Proflavine dihydrochloride)	3.0	1.00
69. 3, 3'-Diamino-4, 4'-dihydroxyarsenobenzene dihydrochloride (arsphenamine; salvarsan)	15.0	2.13
70. 2, 8-Diamino-10-methylacridinium chloride and 2, 8-diaminoacridine (trypaflavin, neutral acriflavine)	18.0	6.26
71. Dibromoxymercurifluorescein sodium (mercurochrome 2% sol.)	10.0	9.43
72. Dibutylamine dimethyl dithiocarbamate	2.0	9.49
73. 3, 5-Dichlorosalicylic acid.	Sat.	2.82
74. 2, 6-Dihydroxy-3-acetoxymercuribenzenesulfonic acid	Sat.	7.00
75. 2, 4-Dihydroxy-3-nitrophenylmercuric chloride	Sat.	6.96
76. a, β-Dihydroxypropylbismuthate	30.0	7.73
77. Dimethylammonium dimethyldithiocarbamate	4.0	7.92
78. 3, 5-Dimethyl-4-chlorophenoxyacetic acid	0.08	3.26
79. 2, 3-Dimethoxy-6-nitro-9-(γ-diethylamino-β-hydroxypropylamino) acridine dihydrochloride + 2-othoxy-6, 9-diaminoacridine lactate + amyl saccharine + sodium biborate (entozon granulate)	2.0	7.50
80. Disodium 3, 3'-diamino-4, 4'-dihydroxyarsenobenzene-N-dimethylenesulfonate (sulfarsphenamine)	6.0	3.23
81. Disodium 2-(4'-di-(carboxymethylenethio)-arsenosoanilino)-s-triazine	20.0	9.23
82. Disodium hydroxymercurisalicyloxyacetate (Mercurosal)	10.0	10.19

*Based on total solution.
†Saturated.

TABLE I (continued)—Compounds Which Killed Trichomonas Foetus in One Minute

Chemical Name	Per Cent	pH of Test Compound
83. Dye characteristics (name withheld)	0.08	7.72
84. Emetine hydrochloride	30.0	1.66
85. Eosine Y	0.8	7.86
86. Ethylhydrocupreine hydrochloride (optochin hydrochloride)	3.0	6.33
87. Ethyl mercury phosphate (lignasan)	0.005	8.05
88. Ferric ammonium sulfate (iron and ammonium alum)	12.0	2.00
89. Ferrous ammonium sulfate	15.0	3.92
90. Formaldehyde (formalin)	0.2	6.04
91. Formic acid	0.05	3.20
92. Glycerine (glycerol)	28.0	6.13
93. Green soap (tincture)	0.05	8.86
94. Hexamethylenamine (methenamine)	26.0	9.03
95. Hexylresorcinol 1:1000 (S. T. 37 antiseptic sol.)	10.0	4.71
96. Hexylresorcinol 1:1000 + alcohol + water soluble jelly (Caprokol)	7.0	3.33
97. Hydriodic acid (sp. gr. 1.5)	0.6	1.83
98. *m*-Hydroxybenzoic acid	0.2	3.30
99. *o*-Hydroxybenzoic acid (salicylic acid)	0.05	3.09
100. *p*-Hydroxybenzoic acid	0.3	3.39
101. 8-Hydroxyquinoline	1.0	7.07
102. 8-Hydroxyquinoline sulfate (chinosol, oxyquinoline sulfate)	23.0	2.38
103. Hydrobromic acid (sp. gr. about 1.38)	2.0	1.52
104. Hydrochloric acid (sp. gr. 1.19)	0.7	1.08
105. Iodine	0.03	4.24
106. Iodine (5 gr.) + potassium iodide (10 gr.) + water (100 cc.) (Lugol's sol.)	0.05	6.19
107. *iso*-Octylhydrocupreine dihydrochloride (Vuzin)	0.01	4.78
108. Kreso dip	0.05	8.32
109. Lactic acid	0.9	2.50
110. Lead acetate	2.0	5.17
111. "Lorol" pyridinium chloride	0.02	6.36
112. "Lorol" thiomethyl pyridinium chloride	0.01	6.45
113. "Lorol" trimethylammonium chloride	0.04	6.36
114. Magnesium sulfate	22.0	6.57
115. Malachite green	0.03	3.73
116. Malachite green + isopropyl alcohol + acetone + formalin (Margreen)	2.0	5.37
117. Malachite green + salicylic acid + tannic acid + isopropyl alcohol (Margreen astringent)	2.0	3.00
118. Mandelic acid	0.3	2.80
119. Manganese sulfate	25.0	5.36
120. 2-(*p* - *tert* - Octylphenoxyethoxyethyl) - mercaptoimidazoline hydrochloride	0.03	5.51
121. Mercuric acetate	0.001	8.36
122. Mercuric bromide	0.005	7.13
123. Mercuric chloride	0.001	6.08
124. Mercuric iodide	0.001	6.02
125. Mercuric nitrate	0.005	7.13
126. Mercuric oxide (red)	0.003	6.95
127. Mercuric sulfate	0.005	7.20
128. Mercurous nitrate	0.3	2.99
129. Mercurous sulfate	0.03	7.07
130. Mercury oxycyanide	0.01	8.80
131. Methylene blue	12.0	2.00
132. *o*-Methoxybenzoic acid	0.4	3.00
133. Monochloracetic acid	0.08	2.86
134. Myristyl gamma picolineum chloride (Quatresin sol. 1:1000)	5.0	5.61
135. Myristyl gamma picolineum chloride + isopropyl alcohol (Quatresin tincture 1:500)	3.0	6.00
136. *N* - (*p* - *tert* - Octylphenoxyethoxyethyl) merpholine hydrochloride	0.08	5.20
137. Nickel (ous) chloride	10.0	6.36
138. 4 - Nitro - anhydro - hydroxy - mercury - orthocresol (Metaphen 1:2500)	3.0	9.60
139. *o*-Nitrobenzoic acid	0.1	2.79
140. *o*-Phenylphenol + isopropyl alcohol + soap (Aseptogen)	0.05	7.04
141. Oxalic acid	3.0	1.36
142. *p* - *tert* - Octylphenoxyethoxyethyldimethylbenzylammoniumchloride (Phemerol)	0.02	6.43
143. Penicillin‡	8.0	7.30
144. Penta-and hexamethylpararosaniline chlorides (Gentian violet, medicinal)	0.04	5.89
145. Phenobarbital sodium	14.0	9.24
146. Phenol (carbolic acid)	2.0	5.88
147. Phenylazodiaminopyridine hydrochloride (Pyridium)	0.08	4.30
148. Phenylmercuric acetate	0.001	8.54
149. Phenylmercuric acetate 1:1000 + hexyl-m-cresol (Bimerphen antiseptic sol.)	0.2	6.09
150. Phenylmercuric acetate 1:1000 + hexyl-m-cresol + alcohol (Bimerphen antiseptic tincture)	0.2	6.03
151. Phenylmercuric borate	0.001	6.44
152. Phenylmercuric chloride	0.001	8.30
153. Phenylmercuric hydroxide	0.001	8.23
154. Phenylmercuric lactate	0.01	6.40
155. Phenylmercuric nitrate	0.001	7.75
156. Phenylmercuric nitrate 1:24,000 + pectin jelly (Merpectogel)	8.0	3.72
157. Phenylmercuric nitrate 1:1000 + isopropyl alcohol + acetone (Phenylmerc)	0.3	7.80
158. Phosphoric acid (conc. about 85%)	1.0	1.92
159. Picric acid	0.4	2.00
160. Pine oil disinfectant	0.08	7.26
161. Polymerized disulphonicdioxydimethyldiphenylmethane acids (sol. Negatan)	2.0	1.96
162. Potassium acid phthalate	5.0	4.10
163. Potassium bromate	10.0	7.30
164. Potassium chlorate	10.0	6.62
165. Potassium chromate	8.0	8.29
166. Potassium dichromate	8.0	4.62
167. Potassium ferricyanide	13.0	6.81
168. Potassium ferrocyanide	18.0	8.62
169. Potassium hydroxide (caustic potash)	0.3	11.34
170. Potassium iodide	3.0	6.36
171. Potassium mercuric iodide	0.001	6.63
172. Potassium permanganate	0.06	6.33
173. Potassium persulfate	7.0	2.25
174. Propylene glycol	45.0	6.13
175. Pyridylmercuric lactate	0.003	7.56
176. Pyrogallic acid	3.0	6.20
177. Quinine dihydrochloride	3.0	6.48
178. *S* - (*p* - *tert* - Octylphenoxyethoxyethyl) isothiourea hydrochloride	0.08	5.78
179. Saponin	0.08	6.15
180. Salicyloxyacetic acid	0.3	2.50
181. Salicylaldoxime	0.4	6.49
182. *sec*-Amyltricresols + *o*-hydroxyphenylmercuric chloride + acetone + alcohol (Mercresin tincture)	0.4	5.89
183. Silver nitrate	3.0	6.22
184. Silver nucleinate (mild silver protein)	1.0	10.0
185. Silver protein (strong)	0.8	10.32
186. Sodium acetate	25.0	8.01
187. Sodium 4-acetonoxybenzene arsonate	15.0	6.50

‡ 80,000 units per cubic centimeter.

TABLE I (continued)—Compounds Which Killed Trichomonas Foetus in One Minute

Chemical Name	Per Cent	pH of Test Compound
188. Sodium 3-amino-4-β-hydroxyethoxybenzenearsonate.	20.0	6.83
189. Sodium 3-amino-4-β-hydroxypropoxybenzenearsonate	17.0	7.03
190. Sodium α-aminodiphenyl acetate	10.0	9.26
191. Sodium benzoate	6.0	7.50
192. Sodium bicarbonate	6.0	6.17
193. Sodium borate	12.0	9.56
194. Sodium bromide	10.0	5.39
195. Sodium carbonate	4.0	10.52
196. Sodium cobalt nitrite	1.0	4.25
197. Sodium 3,3'-diamino-4,4'-dihydroxyarsenobenzene-N-methylenesulfoxalate (neoarsphenamine)	14.0	5.53
198. Sodium ethylmercurithiosalicylate (Merthiolate 1:1000)	2.0	7.46
199. Sodium furoate	20.0	9.70
200. Sodium glycocholate and sodium taurocholate (ox bile salts)	10.0	6.92
201. Sodium hexamethylene dithiocarbamate	6.0	8.68
202. Sodium hydroxide (caustic soda)	0.1	11.06
203. Sodium 4-β-hydroxyethoxybenzenearsonate	15.0	6.50
204. Sodium 4-γ-hydroxypropoxybenzenearsonate	3.0	2.66
205. Sodium hypochlorite (powder)	0.01	9.54
206. Sodium hypochlorite (3.5%) (B-K liquid)	0.3	8.38
207. Sodium hypochlorite + sodium hydroxide + sodium chloride (Zonite)	0.3	7.77
208. Sodium iodide	8.0	8.77
209. Sodium laurylsulfate	0.02	8.10
210. Sodium 3-methylbicyclo (2, 2.1)-5-heptene-2-carboxylate	10.0	9.37
211. Sodium-methylene-sulfonamino-hydroxy-phenylarsonate (phenarsone sulfoxylate; 1 Gm. Aldarsone)	10.0	7.03
212. Sodium 3-methyl-2-norcamphanecarboxylate	10.0	9.65
213. Sodium molybdate	15.0	7.17
214. Sodium N-phenylglycine-Amide-p-arsonic acid (tryparsamide)	16.0	6.29
215. Sodium nitroferricyanide (sodium nitroprusside)	10.0	8.00
216. Sodium o-iodohippurate (Hippuran)	18.0	8.75
217. Sodium oxymercury-ortho-nitro-phenolate 1:2000 + sodium chloride (Mercurophen)	1.0	6.50
218. Sodium p-ethylmercurithiophenylsulfonate (sulfomerthiolate)	0.001	5.34
219. Sodium p-toluenesulfochloramide (Chlorazene)	0.01	6.71
220. Sodium perborate + sodium borate + sodium bicarbonate + sodium chloride + menthol + thymol + methyl salicylate + eucalyptol + aromatics (Stomaseptine)	3.0	8.68
221. Sodium phosphate (dibasic)	20.0	9.26
222. Sodium phosphate (tribasic)	6.0	11.35
223. Sodium salt of p-carbamino phenyl-arsonic acid (sodium salt of carbarsone)	10.0	6.46
224. Sodium salicylate	8.0	6.36
225. Sodium succinylsulfanilamide	20.0	8.29
226. Sodium sulfosuccinic acid, dioctyl ester (aërosol solution)	0.03	8.10
227. Sodium sulfite	9.0	8.72
228. Sodium thiocyanate	6.0	7.19
229. Sodium thiosulfate (sodium hyposulfite)	15.0	5.92
230. Stannous chloride	0.5	2.40
231. Strontium iodide	3.0	10.89
232. Succinic acid	3.0	2.53
233. Sulfapyridine sodium (monohydrate)	9.0	9.84
234. Sulfosalicylic acid	1.0	1.80
235. Superoxyl	32.0	3.77
236. Tannic acid	9.0	3.29
237. Tartaric acid	10.0	1.86
238. Tetraethylthiuram monosulfide	0.8	7.45
239. Thymol + sodium brom-para-phenyl-phenate + naphthalene + safrol + thymene + terpineol + soap (Therapogen)	0.05	6.99
240. Trichloracetic acid	0.5	2.00
241. Trichloro-*tert*-butylalcohol (chlorotone)	2.0	5.07
242. Triethanolamine	12.0	11.12
243. Trisodium bismuthtrithioglycollate (thio-bismol)	18.0	8.65
244. Tyrothricin	0.03	6.49
245. 2-n-Undecyl-4,5-dihydroimidazole hydrochloride	0.1	6.33
246. Uranium acetate	0.3	4.68
247. Uranium nitrate	0.3	3.80
248. Valeric acid	0.5	3.19
249. Zinc acetate	6.0	6.40
250. Zinc chloride	5.0	6.10
251. Zinc chloride + menthol + oil of cinnamon + formaldehyde + saccharine + oil of cloves + alcohol (Lavoris)	60.0	3.48
252. Zinc sulfate	10.0	2.92

(Continued from page 45.)

hyde, 1.0 per cent urotropin, 1.5 per cent phenol, 0.2 per cent quinine hydrochloride, 1.0 per cent tannic acid, 0.1 per cent entozon, 0.1 per cent methyl violet, 0.2 per cent methylene blue, carbon tetrachloride, oil of eucalyptus, and camphor. Certain compounds immobilized trichomonads in twenty-four hours, such as 0.1 per cent tartar emetic, while 5.0 per cent atoxyl, 5.0 per cent Devegan, 1.0 per cent boric acid, 100 per cent hydrogen peroxide, 0.2 per cent potassium permanganate, 2.0 per cent potassium iodide, 20.0 per cent Pregllosung, 1.0 per cent Rongalit (sodium sulfoxalate and formalin), 1.0 per cent sodium salicylate, 1.0 per cent antipyrin, 0.05 per cent hydrocyanic acid, eosin, trypan blue, and nigrosin had no effect.

Böttcher[6] in an unsuccessful attempt to find a substance which would inhibit *T. foetus* and at the same time not affect the motility or fertility of bull spermatozoa found that dilutions from 1:10 to 1:100,000 of trypaflavin, salvarsan (arsphenamine), flavaden, and balkanol failed to kill the trichomonads. Cialit and Hayem's solution at 1:100-1:1000 dilution immobilized *T. foetus*. His test was conducted by placing 2 drops of infected semen with 1 drop of the drug solution and examining microscopically after intervals of one, two, three, and five minutes.

Morisita[7] tested 13 compounds by adding 1 drop of a suspension of *T. foetus* to 1 drop of

a chemical agent and examined at intervals of five, ten, fifteen, twenty, twenty-five, thirty, and sixty minutes. Ten per cent borax, 1.0 per cent boric acid, and alum had no effect. One per cent potassium permanganate, 2.0 per cent phenol, 0.025 per cent mercuric chloride, 1 x 32 Lugol's solution, 70.0 per cent alcohol, 0.25 per cent calcium chloride, and 1 x 2 ether killed *T. foetus* in five minutes, while 0.5 per cent Rivanol, 1.0 per cent cresol, and 1.0 per cent copper sulfate killed in ten minutes.

Kerr[8] studied the resistance of *T. foetus* to certain therapeutic agents. Dilutions of the chemical agents were made with physiological saline or 1.0 per cent peptone saline solution. A 0.1 cc. suspension of 100,000 organisms per cc. was added to 10 cc. of each chemical and examined microscopically at one and five minutes for motility. In saline, the following results were noted: 0.1 per cent acriflavine, 0.01 per cent brilliant green, 0.1 per cent gentian violet, 2.0 per cent lactic acid, 0.01 per cent Merfenil, and 0.001 per cent iodine immobilized *T. foetus* in one minute. With the peptone saline solution the compounds were less effective.

Okljesa and Zaplatic[9] reported on the resistance of *T. foetus* to various uterine antiseptics, but the reference was not available.

A standardized *in vitro* technique for testing the trichomonacidal property of pharmaceuticals utilizing bacteria-free *T. foetus* has been recorded[10]. A partial list of 67 compounds was reported. The details of the test are given below under materials and methods.

MATERIALS AND METHODS

The test consists of introducing 1 cc. of *T. foetus*, suspension into a standard volume of 4 cc. of drug solution. The organisms are suspended in the liquid portion of 24- to 48-hour, bacteria-free *T. foetus* cultures composed of buffered saline citrate solution with 5.0 per cent bovine serum.[11] The number of trichomonads was determined by hemacytometer count and diluted with 0.7 per cent saline in order to obtain a standard suspension of one million organisms per cubic centimeter. Control suspensions were made in a similar manner. *T. foetus* used in this work was a strain isolated by Morgan and Wisnicky[12] and subsequently studied by Morgan.[13]

TABLE 2—Saturated Solutions of Compounds Which Did Not Kill Trichomonas Foetus in Ten Minutes

Chemical Name	pH of Test Compound
1. Acetylamino-hydroxyphenylarsonic acid (Stovarsol; acetarsone)	3.40
2. Acetylamino-hydroxyphenylarsonic acid + boric acid + hydrolyzed carbohydrates (Devegan)	3.60
3. Alcohol, amyl iso (fusel oil)	6.68
4. 2-Amino-4-(4'-arsenosoanilino) pyrimidine	7.06
5. Ammonium oxalate	6.76
6. Antimony potassium tartrate (tartar emetic)	4.13
7. Arbutin	6.26
8. 2-(4'-Arsenosoanilino)-4, 6-diamino-s-triazine	8.17
9. 2-(5'-Arsono-2'-hydroxyanilino)-4, 6-diamino-s-triazine hydrochloride	2.53
10. 4-Biguanidinobenzenearsonic acid	6.76
11. Bismuth oxyiodide	5.88
12. Boric acid	4.17
13. 3-*n*-Butylbenzothiazoloneimide	8.13
14. Camphor	5.92
15. p-Carbamidophenyl arsene oxide	3.06
16. p-Carbaminophenyl-arsonic acid (carbarsone)	3.06
17. Carbon disulfide	8.06
18. Carbon tetrachloride	7.54
19. *m*-Chlorobenzoic acid	4.06
20. Chloroform	7.66
21. Chlorohydroxyquinoline	7.72
22. 5-Chloro-7-iodo-8-hydroxyquinoline (Vioform)	7.53
23. 4-Cyanaminobenzenearsonic acid	3.00
24. 4, 4'-Diamidinodiphenoxypropane (Propamidine)	7.00
25. Dichlorodiphenyltrichlorethane (DDT)	8.06
26. 1, 2-Dihydroxyanthraquinone (Alizarin)	7.90
27. 5, 7,-Diiodo-8-hydroxy-quinoline (Diodoquin)	7.66
28. 5, 7,-Diiodo-8-hydroxy-quinoline (Diodoquin) + boric acid + lactose + anhydrous dextrose (Floraquin)	4.30
29. 3, 5-Dimethyl- 4 -chloro- 2 -morpholinomethylphenol	8.03
30. 3, 5-Dimethyl-4-chlorophenol	7.80
31. Diphenylacetic acid	6.80
32. Diphenylamine	8.29
33. Ether (anesthesia)	8.20
34. Ethyl hydantoinate	5.83
35. Glutamic acid	3.30
36. *o*-Hydroxydiphenyl, o-phenylphenol	7.06
37. 8-Hydroxyquinoline benzoate (oxyquinoline benzoate)	4.26
38. 7 - Iodo - 8 - hydroxyquinoline - 5 - sulfonic acid (chiniofon; yatren)	6.53
39. Lithium carbonate	11.03
40. Mercurous chloride (calomel)	7.97
41. 6-Methoxy-8-nicotinylaminoquinoline	7.72
42. Methyl orange	9.14
43. Methyl salicylate (oil of wintergreen)	6.18
44. Methylenedisalicylic acid	4.35
45. Naphthalene	6.17
46. Phenosafraine	3.80
47. Phenothiazine (thiodiphenylamine)	8.12
48. Phenyl salicylate (salol)	8.40
49. Phthalic anhydride	4.00
50. Phthalylsulfathiazole (S-36)	4.80
51. Silver iodide	8.17
52. Sodium 4, 4'-diaminodiphenylsulfon-2-sulfonacetamide	7.63
53. Sodium methylenesulfonaminohydroxyphenylarsonate (Aldarsone) + kaolin	7.40
54. Sodium oxalate	7.46
55. Sodium pyrophosphate	9.78
56. p-Stibanilic acid	5.56
57. Succinylsulfanilamide	3.90
58. Sulfadiazine	5.68
59. Sulfaguanidine	6.15
60. Sulfamerazine	6.18
61. Sulfamethazine	5.96
62. Sulfanilamide	5.42
63. Sulfanilic acid	2.56
64. Sulfathiazole	5.23
65. Thiosalicylic acid	3.77
66. Thymol	6.66
67. Thymol iodide	8.00
68. Toluol (toluene)	6.03
69. 3, 4, 5-Trihydroxybenzoic acid (gallic acid)	3.07
70. Tri-iodomethane (iodoform)	6.32
71. α-Ureidodiphenylacetic acid	3.65

Various dilutions of the test solutions or suspensions were made up in small, cotton-plugged Erlenmyer flasks and pipetted aseptically to small sterile tubes. Sterile 0.7 per cent saline was used in making the dilutions, as this was shown by Andrews and Lyford[14] and Morgan[15] to be the optimum concentration of saline for motility of *T. foetus*. Occasionally, sodium hydroxide and propylene glycol were used with drugs which were not soluble in saline. The two ingredients were thoroughly mixed and the tubes placed in a water bath at 37 C. At intervals of one, two, three, five, and ten minutes the trichomonads were pipetted on special Boerner[16] slides with 12-mold pressed raised rings and examined with the low power of the microscope. As soon as the organisms were immobilized, 1 cc. of material was pipetted into regular culture medium. The subcultures were examined microscopically at one-half and one hour for motility of *T. foetus*. All material was handled aseptically.

TABLE 3—One Per Cent Suspensions of Compounds Which Did Not Kill Trichomonas Foetus in Ten Minutes

Chemical Name	pH of Test Compound
1. Alcohol, capryl (caprylic alcohol; octylic alcohol)	7.00
2. Cetylpyridinium chloride (.5%) + boric acid + kaolin + dextrose (Ceepryn vaginal powder borated)	6.52
3. Oil eucalyptus	7.56
4. Oil thuja	5.00
5. Oil thyme	7.72
6. Oleic acid	7.60

TABLE 4—Compounds Which Killed Trichomonas Foetus after Varying Exposures

Chemical Name	Per cent	pH of test compound	Exposure time in minutes
1. Hydrogen peroxide	100	3.50	2
2. m-Cresylacetate (cresatin)	100	7.00	5
3. Silver chloride-thiourea complex salt + 3-chloro-4-hydroxy diphenyl + osmo-kaolin (Povage)	1 Susp.§	6.72	2
4. Silver colloidal sulfathiazole + beta lactose + kaolin (colloidal silver sulfathiazole compound powder)	Sat.	6.72	5
5. Silver colloidal sulfathiazole 25% (colloidal silver sulfathiazole compound)	Sat.	7.23	2
6. Sodium perborate	Sat.	9.93	5

§ Suspension.

Failure of the trichomonads to show motility after transfer to the medium indicated that the test mixture was lethal. Consequently, this also indicated that the organisms had been killed by exposure for a definite time interval to a given percentage dilution of a chemical agent.

The effective concentration of the test chemical was arbitrarily accepted as the dilution which killed *T. foetus* in one minute. The percentage of the compound was increased until this was accomplished. Compounds which did not meet this requirement were considered ineffective as measured by the *in vitro* test. It was realized that some of the compounds may have had lethal effects on *T. foetus* at longer periods of exposure. Long intervals of exposure in high dilutions may affect some enzyme system while a short exposure would have no effect.

The pH determinations of the test mixtures were made with a Coleman 200 electrometer. The hydrogen-ion concentration is expressed as pH. In the computation of the means, the pH values were converted into hydrogen-ion concentration expressed as gram ions per liter. The means were then reconverted into pH values.

RESULTS

All tests were run in triplicate at the same final dilution. The data obtained are tabulated as follows: (table 1) compounds dissolved in 0.7 per cent saline which killed *T. foetus* in one minute; (table 2) saturated solutions of compounds which did not kill *T. foetus* in ten minutes; (table 3) 1.0 per cent suspensions of compounds which did not kill *T. foetus* in ten minutes; (table 4) compounds which killed *T. foetus* after varying exposures; (table 5) solvents combined with compounds which killed *T. foetus* in one minute; and (table 6) sol-

TABLE 5—Various Solvents Combined with Compounds Which Killed Trichomonas Foetus in One Minute

Chemical Name	Solvent	Per cent	pH of test compound
1. Chlorothymol	Propylene glycol (5%)	0.1	6.19
2. *m*-Dihydroxydi-*sec*-hexylbenzene (Dihexylin)	Propylene glycol (5%)	0.1	6.22
3. Octyloxyacetic acid	Sodium hydroxide (.005%)	0.5	3.58
4. 4-Oxalaminobenzenearsonic acid	Sodium hydroxide (.005%)	3.0	3.00
5. Phenylethylglycine	Sodium hydroxide (.005%)	3.0	1.89
6. dl-Pyrrolidone carboxylic acid	Sodium hydroxide (.005%)	8.0	2.17
7. Silver chloride-thiourea complex salt (argenitis)	Distilled water	13.0	6.30
8. Sodium chloride	Distilled water	6.0	5.35

vents combined with compounds which did not kill *T. foetus* in one minute.

DISCUSSION

The compounds tested, which demonstrate trichomonacidal power in high dilutions *in vitro*, as presented in the above tables, should be examined further for toxicity and action on mucous membranes. The most promising compounds should be studied with a standardized, controlled, *in vivo* test for their therapeutic action.

TABLE 6—Various Solvents Combined with Compounds Which Did Not Kill Trichomonas Foetus in One Minute

Chemical Name	Solvent	Per cent	pH of test compound
1. 4-Arsenosobenzoic acid	NaCl (0.7%)	Sat.	4.17
2. 3-Biguanidino-4-β-hydroxyethoxybenzenearsonic acid	NaOH (.005%)	10.0	....
3. 4, 4'-Diamidinostilbene (Stilbamidine)	NaCl (0.7%)	25.0	7.20
4. 4-/B-(b'-hydroxyethoxy)-ethoxybenzenearsonic acid	NaCl (0.7%)	15.0	....
5. 6-Methoxy-4-γ-diethylaminopropylaminoquinaldine	NaCl (0.7%)	6.0	6.10
6. Quinine sulphate	NaCl (0.7%)	5.0	7.40
7. Water (distilled)		100.0	8.06

SUMMARY

A total of 350 compounds has been tested for trichomonacidal properties by a standardized *in vitro* technique. The lowest concentration which killed *Trichomonas foetus* in one minute is taken as the effective concentration. The data summarized in tables 1 to 6 record results which may offer useful information for a more satisfactory therapeutic agent than is now available for the treatment of bovine trichomoniasis.

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The Influence of Protein Diets on Selenium Poisoning. I.

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THE WIDESPREAD distribution of selenium in the western states and its relation to livestock poisoning have been observed by many investigators.[1,4] Acute, subacute, and chronic selenium poisoning are responsible for severe losses in horses, cattle, hogs, sheep, and chickens. The economic consequences have been tragic in localities where the soil contains appreciable amounts of available selenium. Investigations to counteract the toxic effects of selenium up to the present time have been unsuccessful. Moxon[5] found that arsenic had a beneficial effect when fed to pigs receiving a seleniferous ration. Moxon *et al.*,[6] in a later study, reported that steers grazed on a seleniferous range and offered 25 p.p.m. of arsenic mixed with salt made better gains, and the incidence of symptoms of selenium poisoning was reduced by this treatment. Studies dealing with the effect of high protein diets upon selenium poisoning are contradictory. Schneider[7] observed that the addition of various quantities of cystine as a supplement to a basal low protein diet neither delayed nor arrested the lethal action of dietary inorganic selenium. The experiments of Lewis, Schultz, and Gartner[8] suggested that the dietary protein and protein supplements modified the toxicity of inorganic selenium compounds. Smith and Stohlman[9] obtained good protection by use of wheat and corn proteins in rat rations. However, Moxon[10] found that in dogs the most severe poisoning occurred on rations containing large amounts of wheat and corn protein. These reports emphasize the discrepancies in the results obtained in the different species of animals when various protein diets were added to selenium. Since selenium poisoning is a serious hazard to livestock, experimental work with farm animals is needed before preventive measures can be worked out. With this aim in mind, our experiments were designed to reproduce experimentally, as nearly as possible, the actual field conditions and to investigate the effects of various protein diets upon accumulative selenium poisoning. We also attempted to work out a correlation between food intake and the subtoxic, toxic, and lethal doses of selenium in sheep. The results of these investigations will be presented in this paper. The results dealing with the chemical changes in the tissues and the distribution and elimination of selenium in the different organs will be presented in subsequent papers.

TABLE I—The Composition of the Different Protein Diets

High Protein Diet (%)	Medium Protein Diet (%)	Low Protein Diet (%)
30 ground yellow corn	12.5 ground yellow corn	25 dried starch waste
25 linseed meal	16.0 linseed meal	25 wheat straw
18 cottonseed meal	10.0 cottonseed meal	19 molasses
13 wheat bran	12.5 wheat bran	25 cottonseed hull
10 molasses	20.0 molasses	2 corn oil
4 mineral mixture*	4.0 mineral mixture*	4 mineral mixture*
	25.0 wheat straw	

* Mineral mixture: 40 per cent ground limestone, 40 per cent disodium phosphate, 20 per cent iodized salt.

METHODS

Yearling ewes were used in this experiment. Their average weights varied from 75 to 80 lb.

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at the beginning of the experiment. The groups were balanced as nearly as possible with regard to weight. The animals were divided into four groups of five sheep. Group 1 received a diet containing 20.0 per cent digestible protein*; group 2 was fed a diet containing 12.0 per cent digestible protein†; group 3 was given a diet containing 1.5 per cent digestible protein‡; and group 4, the control animals, were on a selenium-free ration. The compositions of the different diets are given in table 1. The selenium was given by drenching. Water extract of the seleniferous plant, *Atriplex canescens*, was used as a source of organic selenium. The concentration of selenium in the extract was determined by the official method of Association of Official Agricultural Chemists.[11] However, we found that whether we used the organic selenium or the inorganic selenium of sodium selenite (Na_2SeO_3), we could produce acute intoxication with the same dose when the selenium was administered orally. Therefore, toward the end of the experiment we used the inorganic selenium only. Martin[12] found that rats consuming seleniferous buckwheat plants as part of their diet responded in the same manner as those that received an equivalent amount of inorganic selenium in their diet.

Each group was started with 10 mg. of selenium daily, and increased to 15 and 20 mg. When all the animals (from each group) showed symptoms of intoxication, the drenching was discontinued in two sheep, and in three sheep, the dose was increased to 30 mg. daily. The selenium feeding was discontinued in order to study the duration of toxic symptoms and to study the elimination of selenium from the different organs. Blood samples were taken at regular intervals from each animal. The selenium in the blood was determined by the method of Klein[13].

*Referred to as high protein diet.
†Referred to as medium protein diet.
‡Referred to as low protein diet.

TABLE 2—Relation of Food Consumption to Selenium Intoxication

High Protein				Medium Protein				Low Protein			
Selenium		Average food		Selenium		Average food		Selenium		Average food	
mg./day	days	Consumed Gm./day	Decrease (%)	mg./day	days	Consumed Gm./day	Decrease (%)	mg./day	days	Consumed Gm./day	Decrease (%)
0	0	1000(5)*	....	0	0	1000(5)	....	0	0	1000(5)	....
10	21	780(5)	22.0	10	21	940(5)	6.0	10	21	560(5)	44.0
15	6	700(5)	30.0	15	6	669(5)	34.0	15†	6	450(5)	55.0
20†	25	500(5)	50.0	20	24	481(5)	51.9	20	7	210(3)	79.0
30	27	250(3)	75.0	30	27	225(3)	77.5	20	12	50(3)	95.0
30	13	80(3)	92.0	30	13	100(3)	90.0	..	..	.....	....

* Number of animals.
† Symptoms of intoxication occurred.

RESULTS

The animals were placed on high and medium protein diets for one month before the selenium feeding was begun. The low protein diet was given to the animals for two months before selenium feeding was started in order to obtain a minimum maintenance level in all animals. The daily food consumption in each group was 1,000 Gm. per sheep before selenium feeding was started.

Table 2 gives the results obtained. The food consumption in the high and medium protein-diet groups was reduced 22.0 and 6.0 per cent, respectively, after feeding with 10 mg. of selenium for twenty-five days, and in the low protein-diet group the reduction was 44.0 per cent. As the amount of selenium was increased there was a corresponding decrease in the food consumption. When the food intake decreased 50.0 per cent or more, the animals showed symptoms of acute intoxication. Toxic symptoms

TABLE 3—The Increase of Food Intake After Selenium Administration Was Discontinued

High Protein			Medium Protein			Low Protein		
Total Se given (mg.)	Se discontinued (days)	Average food consumed* (Gm.)	Total Se given (mg.)	Se discontinued (days)	Average food consumed (Gm.)	Total Se given (mg.)	Se discontinued (days)	Average food consumed (Gm.)
800	..	250 (2)	800	..	225 (2)	360	..	210 (2)
...	1	250 (2)	...	1	225 (2)	...	1	210 (2)
...	10	800 (2)	...	10	600 (2)	...	10	400 (2)
...	26	1000 (2)	...	26	800 (2)	...	27	800 (2)

* Average number of animals indicated by the parentheses.
Se = selenium.

appeared in the high and medium protein-diet groups after the administration of 20 mg. of selenium for twenty-five days, and in the low protein group after 15 mg. for six days. It is evident from these results that in the high and medium protein-diet groups a higher concentration of selenium was necessary to produce intoxication than in the low protein group. In the high and medium protein-diet groups, death occurred after feeding 20 mg. of selenium daily for forty days, with a decrease of food intake of 90.0 per cent or over. In the low protein group 20 mg. of selenium daily for nineteen days was fatal, with a reduction of food intake of 95.0 per cent.

TABLE 4—Increase of Selenium in the Blood in the Groups Receiving Selenium in the Diet

Blood specimen	High Protein Diet		Medium Protein Diet		Low Protein Diet	
	Total Se Administered	Se in Blood	Total Se Administered	Se in Blood	Total Se Administered	Se in Blood
	mg.	p.p.m.	mg.	p.p.m.	mg.	p.p.m.
Selenium given for 14 days....	140	0.690	140	0.789	75	0.985
Selenium given for 44 days....	800	1.135	800	1.598	360*	1.388
Selenium given for 64 days....	1,200	1.165	1,200	1.456	632†	2.986
Selenium given for 87 days....	1,660	2.638	1,660	2.025	...	.....
Selenium given for 116 days....	1,922	2.51	1,922	2.092	...	.....
Control	0	0.586	0	0.723	0	0.570

* Selenium was given in 34 days.
† Selenium was given in 46 days.

Table 3 gives the results obtained after the selenium feeding was discontinued in the intoxicated animals. The symptoms of intoxication gradually decreased and the appetite of the animals increased after a few days. Within ten days the high protein group consumed, on the average, 800 Gm., and the medium protein-diet group 400 Gm. The return to the normal food intake was much slower in the low and medium protein groups than in the high protein group. This variation may be due to the difference in the palatability of the food.

TABLE 5—Decrease of Selenium in Blood After Selenium Feeding Was Discontinued

Blood specimens taken	High Protein Diet		Medium Protein Diet		Low Protein Diet	
	Total Se given	Se in blood	Total Se given	Se in blood	Total Se given	Se in blood
	mg.	p.p.m.	mg.	p.p.m.	mg.	p.p.m.
Selenium fed for 44 days.....	800	1.135	800	1.598	360*	1.388
Selenium discontinued for 14 days.....		1.503	...	1.67	...	2.024
Selenium discontinued for 32 days.....		1.889	...	1.926	...	1.564
Selenium discontinued for 61 days.....		0.42	...	0.56	...	0.842

* Selenium was given for 34 days.

The accumulative effect of selenium plays an important part in selenium poisoning. One single dose of 15 mg. or 20 mg. of selenium was not intoxicating to the sheep. However, the continued administration of the same dose produced toxic symptoms. Toxic symptoms occurred in the high and medium protein-diet groups after the animals received 800 mg. of selenium, and in the low protein group 360 mg. of selenium. In the low protein group, the intoxicating dose and the fatal dose were very close, while in the high and medium protein groups the range was much greater. No evidence of tolerance to selenium intoxication was observed as has been reported by Modica[14], but rather an accumulative effect was observed. Our conclusions are in agreement with the observations of Smith, Stohlman, and Lillie[15].

Table 4 gives the increase of selenium in the blood in the three groups during selenium feeding. The increase of selenium in the blood was a gradual process. The animals of the high and medium protein groups received 800 mg. of selenium in forty-four days, and the blood level rose to 1.135 and 1.598 p.p.m., respectively. In the low protein group, the blood level of 1.388 p.p.m. was reached when 360 mg. of seleni-

um was given in thirty-four days. Symptoms of intoxication appeared in all groups when the selenium in the blood increased between 1.2 to 1.5 p.p.m. and the feeding of the selenium was continued. The selenium in the blood of the low protein group increased in twelve days from 1.388 to 2.986 p.p.m., at which time the animals died from selenium poisoning. The increase in the high and medium protein-diet groups from 1.135 and 1.598 to 2.51 and 2.092 p.p.m. had taken place in seventy-two days. However, in both groups the blood contained over 2.00 p.p.m. of selenium for twenty-nine days before death occurred. As can be seen from the tables, there was a more rapid rise of selenium in the blood in the low protein-diet group than in the higher protein groups. In studying the elimination of the selenium in the urine in the different groups, we found it to be approximately the same in all groups. Therefore, we assume that the difference in the resistance of the higher protein groups was associated with the protein content of the tissues and their ability to store a much higher concentration of selenium without cellular injury.

Table 5 gives the results obtained after selenium feeding was discontinued in the different groups. These results indicate that after selenium feeding ceased, there was a release from the tissues of the stored selenium with the resulting increase of selenium in the blood. The blood maintained a high level of selenium for thirty-two days, after which there was a gradual decrease to a low level in all groups.

SUMMARY

The feeding of high, medium, and low protein diets to sheep indicated that the high and medium protein diets afforded more protection against selenium poisoning than the low protein diet. There was no essential difference in protective action between the high and medium protein diets when protection was based upon the duration of life.

The intake of 10 mg. of selenium daily did not produce intoxication in the animals. However, it reduced the food intake in all groups. This suggests that a seleniferous area which supplies 10 mg. of selenium daily in the food of the animals would lower the nutritional state of the animals unless the food is of high quality and thus makes up for the lowered food intake.

The intoxicating dose in the low protein group was 15 mg. of selenium given for six days, while in the higher protein groups 20 mg. of selenium was administered for twenty-five days before producing toxic symptoms. The fatal dose in the high and medium protein-diet groups was 30 mg. of selenium for thirty days, and in the low protein-diet group, 20 mg. for thirteen days.

Recovery from acute intoxication was slower in the low protein and medium protein groups than in the high protein group, judging from the food intake.

Selenium content of the blood in the low protein-diet group reached a higher level with a lower selenium intake than in the groups which received high and medium protein diets.

Symptoms of intoxication occurred in all groups when the blood contained between 1.2 to 1.5 p.p.m. selenium and selenium feeding was continued.

Death occurred in all groups after the concentration of selenium in the blood was increased above 2 p.p.m. of selenium and selenium feeding was continued. Higher protein groups maintained a high blood level of selenium for a longer time than did the low protein group, before death occurred.

With the cessation of selenium feeding the blood selenium showed an increase first, then a gradual drop to less than 1.0 p.p.m. of selenium.

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The Influence of Protein Diets on Selenium Poisoning

II. The Chemical Changes in the Tissues Following Selenium Administration

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STUDIES DEALING with the changes in the chemical composition of the tissues in selenium poisoning are few or are completely lacking. Franke and Potter[1] observed that progressive anemia developed in chronic selenium poisoning. Anderson and Moxon[2] studied the changes in the blood picture in acute selenium poisoning. They reported a marked increase in hemoglobin, hematocrit, and blood sugar, and a decrease in inorganic phosphorus, nonprotein nitrogen, and ascorbic acid. Rhian and Moxon[3] reported that chronic selenium poisoning had little or no effect on the commonly determined constituents of the blood except that the hemoglobin and possibly nonprotein nitrogen decreased and the phosphatase activity increased. Svirbely[4] reported a decrease in the vitamin C content of the liver and adrenals, in selenium poisoning. No investigations relating to the chemical changes in other tissues besides blood are available at the present time. Miller and Whipple[5] found that protein-depleted dog livers were deficient in nitrogen and sulfur, and these dogs were susceptible to injurious agents—in particular chloroform. The characteristic lesion of chloroform and selenium poisoning is hepatic cellular necrosis. Therefore, it seemed appropriate to investigate whether any correlation between the liver injury in selenium poisoning and the depletion of protein and sulfur exists. The purpose of this paper is to present the results obtained on the analysis of the different tissues in selenium poisoning.

Approved for publication by the director of Wyoming Experiment Station, University of Wyoming, Laramie.

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EXPERIMENTAL PROCEDURE

Experimental animals and their diets were given in the preceding paper. Blood samples were taken at regular intervals from the jugular veins of the animals. Analysis of the blood included the following determinations: vitamin A by the method of Kimble[6]; ascorbic acid by the method of Mindlin and Butler[7]; total protein by micro-Kjeldahl method; photoelectric colorimetric adaptations of nonprotein nitrogen by the method of Folin and Wu[8]; inorganic phosphorus by the method of Fisk and Subbarow,[9] and calcium by the method of Roe and Kahn.[10] The analysis of the tissues included the following determinations: total nitrogen by macro-Kjeldahl method; sulfur by a Parr bomb method; vitamin A by the method of Lewis, Bodansky, Falk, and McGuire,[11] modified so as to use 50 Gm. of liver; ascorbic acid by the method of Hochberg, Melnick, and Oser.[12]

The tissues for the analysis of the sulfur and nitrogen were dried at 70 C. The dried tissues were then ground to powder, mixed, and samples were taken for analysis.

RESULTS

Changes observed in the blood at various intervals of selenium feeding are given in tables 1 and 2. There was a gradual decrease of vitamin A regardless of diets. All diets contained sufficient carotene for the daily requirements of the sheep, which according to Guilbert, Miller, and Hughes[13] was 25 to 30μg per kilogram of body weight. The analysis of the different diets indicated that the low protein diet contained 350μg per cent, the medium 450μg per cent, and the high protein diet 580μg per cent of carotene. We know that selenium is injurious to the liver, and this injury may be responsible for the lack of utilization of carotene and the gradual decrease of vitamin A in the blood. There was a marked decrease of ascorbic acid in the blood. The decrease of ascorbic acid was more rapid than the decrease of vitamin

TABLE 1—Chemical Changes in the Blood, Selenium Administered Until Death

Blood specimens	High Protein Diet					Medium Protein Diet				
	Se fed* (mg.)	Vit. A (mμ%)	Ascorbic acid (mg.%)	Total Protein (mg.%)	N.P.N. (mg.%)	Se fed (mg.)	Vit. A (mμ%)	Ascorbic acid (mg.%)	Total Protein (mg.%)	N.P.N. (mg.%)
Control	0 (3)	32.5	0.6068	7.2	....	0	25.7	0.6198	8.1	....
On diet for 24 days	0 (5)	33.7	0.6939	7.3	38.7	0	26.0	0.5086	8.1	31.4
Se given for 14 days	140 (5)	31.8	0.2906	6.7	50.3	140	29.0	0.2388	7.0	36.4
Se given 44 days	800 (5)	27.7	0.311	4.9	82.9	800	27.0	0.257	4.7	68.2
Se given 64 days	1,200 (3)	29.9	0.294	4.7	77.5	1,200	30.8	0.3435	4.3	47.3
Se given 87 days	1,660 (3)	20.9	0.1545	5.2	104.5	1,660	21.6	0.1276	5.7	65.7
Se given 116 days	1,922 (3)	16.8	0.067	5.5	126.0	1,922	15.6	0.016	5.2	103.4

*Indicates the number of sheep used.

A. Svirbely[4], studying *in vitro* the effect of selenium salts upon ascorbic acid, found that the selenium salts were reduced by ascorbic acid. Our observations indicated that there was a direct relation between the decrease of ascorbic acid and selenium intake. As long as selenium feeding was continued there was a gradual decrease in the ascorbic acid. In addition to the decrease of vitamins, there was a gradual loss of the total protein in all groups. This decrease was more marked in the low protein diet group than in the higher protein diet groups. This difference may be due to the higher protein intake in the latter groups. Increase in the nonprotein nitrogen occurred in all animals before death, and this indicates kidney injury. The gross pathology of the kidneys in our experimental animals showed acute glomerulonephritis, which condition was responsible for the increased nonprotein nitrogen.

Tables 3 and 4 give the changes in the blood which occurred during and after discontinuation of selenium feeding. These results also show the gradual decrease in the vitamin A, ascorbic acid, and total protein during selenium feeding and a gradual increase after selenium was discontinued. The change in the amount of ascorbic acid was very significant. Our present conception that ruminants are able to synthesize enough ascorbic acid without any supplements must be modified, since our observations indicated that selenium destroys the ascorbic acid. Therefore, in chronic selenium poisoning we must consider vitamin C deficiency. The discontinuation of selenium resulted in a gradual increase in the different constituents of the blood, and the blood picture returned to the normal levels. Determinations for calcium, phosphorus, and magnesium were carried out in the blood. There was some fluctuation in the calcium and phosphorus levels at the time when intoxication occurred. However, later there was a return to normal.

Table 5 gives the changes in the chemical composition of the liver in selenium poisoning. There was a decrease in the nitrogen, total protein, sulfur, vitamin A, and ascorbic acid in those animals which died of selenium poisoning. A decrease from 20 to 30 per cent nitrogen occurred in all groups of animals, indicating that depletion of the nitrogen occurred much more rapidly in the low protein-diet group than in the medium and high groups. This suggests that the protective actions of the higher protein diets may be due to the availability of protein and more complete saturation of the liver cells. The defense mechanism of the liver cells in selenium poisoning differs from that suggested by

TABLE 2—Chemical Changes in the Blood, Selenium Administered Until Death

Blood specimens	Low Protein Diet				
	Se fed* (mg.)	Vit. A (mμ%)	Ascorbic acid (mg.%)	Total Protein (mg.%)	N.P.N. (mg.%)
On diet for 64 days	0 (5)	36.0	0.4210	5.5	31.3
Se given 14 days	140 (5)	20.2	0.1413	2.7	36.0
Se given 34 days	360 (5)	27.5	0.208	2.3	47.7
Se given 46 days	532 (3)	13.3	0.2384	...	52.6
Control	0 (3)	26.7	0.6367	7.6	....

*Indicates the number of sheep used.

TABLE 3—Chemical Changes in the Blood During, and After Discontinuation of, Selenium Feeding

Blood specimens	High Protein Diet					Medium Protein Diet				
	Se fed* (mg.)	Vit. A (mμ%)	Ascorbic acid (mg.%)	Total Protein (mg.%)	N.P.N. (mg.%)	Se fed (mg.)	Vit. A (mμ%)	Ascorbic acid (mg.%)	Total Protein (mg.%)	N.P.N. (mg.%)
On diet for 24 days....	0 (2)	32.5	0.5598	6.4	34.2	0	28.5	0.5812	8.4	31.3
Se given 14 days	140	21.4	0.1806	6.8	63.1	140	26.7	0.0	5.1	53.0
Se given 44 days	800	20.6	0.1833	4.0	68.8	800	22.6	0.231	4.6	60.2
Se was discontinued for 14 days	...	19.9	0.3665	5.3	64.1	...	21.8	0.252	...	48.3
Se was discontinued for 32 days	...	22.4	0.4225	7.6	38.8	...	21.9	0.321	5.7	37.9
Se was discontinued for 61 days	...	29.6	0.4425	7.6	38.7	...	20.4	0.350	6.6	40.0
Control	0 (2)	31.1	0.7281	7.3	....	0	27.0	0.5581	8.5	30.1

*Indicates the number of sheep used.

TABLE 4—Chemical Changes in the Blood During, and After Discontinuation of, Selenium Feeding

Blood specimens	Low Protein Diet				
	Se fed* (mg.)	Vit. A (mμ%)	Ascorbic acid (mg.%)	Total Protein (mg.%)	N.P.N. (mg.%)
On diet for 64 days.....	0 (2)	33.6	0.5781	5.4	28.5
Se given 14 days	140	22.8	0.253	5.2	33.5
Se given 34 days	360	15.3	0.391	4.5	80.5
Se discontinued for 10 days.....	...	20.0	0.416	4.1	38.0
Se discontinued for 61 days.....	...	22.4	0.456	4.9	32.1
Control	0 (2)	32.6	0.5702	6.2	....

*Indicates the number of sheep used.

TABLE 5—Changes in the Chemical Composition of the Liver in Selenium Poisoning

Diet	Nitrogen* (Gm.%)	Total Protein (Nx6.25) (Gm.%)	Total Sulfur* (Gm.%)	Vit. A (mμ/Gm.)	Ascorbic acid (mg.%)
High Protein Diet					
1. Selenium given until death.....	9.11 (3)†	56.94 (3)	0.673 (5)	7.83 (3)	7.08 (3)
2. Selenium given for 44 days, then discontinued for 61 days and animal killed.....	10.72 (2)	67.00 (2)	0.776 (2)	21.8 (2)	20.4 (2)
Medium Protein Diet					
1. Selenium given until death.....	8.11 (3)	50.68 (3)	0.659 (3)	8.41 (3)	8.76 (3)
2. Selenium given for 44 days, then discontinued for 61 days and animal killed.....	10.9 (2)	68.13 (2)	0.760 (2)	19.50 (2)	23.6 (2)
Low Protein Diet					
1. Selenium given until death.....	8.13 (3)	50.8 (3)	0.628 (3)	13.20 (3)	10.2 (3)
2. Selenium given for 34 days, then discontinued for 64 days and animal killed.....	10.81 (2)	67.46 (2)	0.821 (2)	18.15 (2)	20.0 (2)
Control	11.9 (5)	74.38 (5)	0.801 (5)	28.97 (5)	19.3 (5)

*The results are given on the basis of dried tissue.
†Indicates the number of animals used.

TABLE 6—Changes in the Chemical Composition of Tissues in Selenium Poisoning

Organs	Selenium Administered Until Death						Selenium Administered for 44 Days and Animals Killed 61 and 64 Days After Selenium was Discontinued					
	Total Nitrogen* Protein Diets						Total Nitrogen* Protein Diets					
	High (Gm.%)		Medium (Gm.%)		Low (Gm.%)		High (Gm.%)		Medium (Gm.%)		Low (Gm.%)	
Heart	6.7	(2)†	11.1	(2)	7.7	(2)	11.1	(2)	11.3	(2)	10.5	(2)
Lungs	11.9	(3)	12.9	(3)	11.5	(3)	12.4	(2)	12.2	(1)	11.7	(2)
Kidneys	10.9	(3)	11.1	(3)	10.3	(3)	10.8	(2)	11.0	(2)	11.3	(2)
Spleen	.....		12.2	(2)	10.1	(1)	12.5	(1)	.....		.....	
	Total Sulfur*						Total Sulfur*					
Heart	0.693	(2)	0.831	(2)	0.737	(3)	0.841	(2)	0.821	(2)	0.878	(2)
Lungs	0.865	(3)	0.803	(3)	0.841	(2)	0.797	(2)	0.760	(1)	0.567	(2)
Kidneys	0.887	(3)	0.845	(2)	0.779	(2)	0.846	(2)	0.855	(2)	0.799	(2)
Spleen	.....		0.891	(2)	1.064	(1)	0.859	(1)	.....		.....	

*The results are given on the basis of dried tissues.
†Indicates the number of tissues used for analysis.

Miller and Whipple[5] in chloroform poisoning, in that the loss of sulfur in selenium poisoning was not as significant as that observed in chloroform poisoning. Nevertheless it was apparent that the loss of protein and probably sulfur from the liver in some manner was associated with the susceptibility of the animals to selenium poisoning. Depletion occurred more readily in the low protein group and the duration of life was shorter. In the high protein groups, the diets supplied a large excess of protein. The duration of life continued as long as the livers were able to utilize the available protein, and protein depletion occurred at a much later time. At the time of death, the livers of all animals showed a decrease in the total protein. Analyses of the livers indicated lowered vitamin A and ascorbic acid content. The loss of vitamins may have been due to either insufficient storage or depletion during selenium toxication.

Table 6 gives the changes in the chemical composition of the other organs in selenium poisoning. The lungs, kidneys, and a limited number of spleens showed no changes in nitrogen and sulfur content. The hearts of the animals on high and low protein diets showed marked decreases in the nitrogen and less marked decreases in the sulfur contents. The loss of the nitrogen may have been responsible for the myocardial degeneration observed in selenium poisoning. According to our results, no change in the chemical composition of the hearts of animals fed with medium protein diets occurred. This difference may have been due to the fact that the high and medium protein groups showed a large amount of fat, while in the medium protein groups the fatty accumulation was much less prominent. Studies to investigate the changes in the fat metabolism in the different organs and its relation to selenium poisoning will be carried out in the near future.

SUMMARY

Selenium poisoning in sheep produced changes in the chemical composition of the different tissues.

There was a gradual decrease in the vitamin A and total protein content of the blood, and a more rapid decrease in ascorbic acid, suggesting that vitamins and protein deficiencies play an important part in selenium poisoning. The deficiency of ascorbic acid in ruminants must be considered in seleniferous areas. An increase of nonprotein nitrogen in the blood was observed in all animals which died from selenium poisoning.

The chemical composition of the livers indicated that selenium poisoning in the sheep produced protein and sulfur depletion in these organs. The increased resistance against selenium poisoning in animals fed high protein diets may be due to the more complete saturation of the liver cells and to the protective action of the protein against the injury of the cell by the toxic action of selenium. A decrease of vitamin A and ascorbic acid was observed in the livers of animals which died of selenium poisoning. The depletion of nitrogen was not restricted to the liver but occurred also in the heart in some groups. The kidneys, lungs, and spleens showed no changes as to the nitrogen, total protein, and sulfur content.

Changes in the chemical composition of the blood, before and after selenium feeding was discontinued, indicated that the decrease of the blood constituents was temporary in nature, and there was a gradual rise toward the normal level after selenium feeding was discontinued.

Chemical composition of the tissues sixty-one to sixty-four days after discontinuation of selenium feeding showed a 10 per cent decrease in the nitrogen content of the liver, but all other organs were normal. This decrease indicated that protein depletion in the liver was a gradual process, and during selenium feeding there was a continued loss of the protein from the liver.

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Salmonella from Galapagos Turtles, a Gila Monster, and an Iguana

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DURING THE PAST three years the writers have examined tissues and stool specimens from 153 animals (21 mammals, 85 birds, and 47 reptiles) on exhibit at the San Diego Zoo. A total of 7 Salmonella types were isolated during this survey. Reports published[1,2] include three of these—*Salmonella typhimurium, Salmonella kentucky,* and *Salmonella panama.* The present paper reports the isolation of *Salmonella sandiego* and *Salmonella newport* from Galapagos turtles, *Salmonella montevideo* from a gila monster and *Salmonella manhattan* from an iguana, included in the survey.

Galapagos Turtles.—Two of these animals died without symptoms being reported, but on autopsy they were found to be emaciated, and the abdominal cavities were filled with a fetid, sanguineous fluid. Other autopsy findings were flabby hearts, enlarged bronze-colored livers which were very friable, and enlarged spleens. With the exception of a small amount of formed feces in the rectum of 1 of them, the digestive tracts were empty. Postmortem decomposition prevented an accurate examination of the intestines, but there was marked congestion of the ileums. The mucous membrane of the bladder of 1 was markedly thickened, and the small amount of contents was viscous and dark brown. There was also marked hepatization of the lungs in this last animal.

Bacteriological examination of the organs resulted in the isolation of Salmonella from the liver, spleen, lungs, and intestine of one; and from the abdominal fluid, intestine and bladder of the other. When antigenically typed, the cultures of the first animal proved to be *S. newport* (VI, VIII: e,h: 1,2,3). The others proved to be *S. sandiego* (IV, V, XII; e,h; enz$_{15}$).

After the second isolation was made, 13 urine and fecal samples, collected from the exhibit yard or from individual animals in the yard, were seeded into tetrathionate broth, Difco, plus 1 to 50,000 brilliant green. From these enriched cultures one strain of *S. sandiego* was isolated from the desoxycholate plates seeded from them. It came from a freshly voided urine specimen of a tortoise. Three weeks later, ten more specimens were collected from the same yard. Nine of these were obtained by swabbing the cloaca of individuals with a sterile cotton swab, while one was obtained from a fresh stool. No Salmonella were obtained from these ten specimens. These did not, however, include a specimen from the positive animal of the previous trial.

Gila Monster.—On Oct. 3, 1944, a captive female gila monster (*Heloderma suspectum*) died after several weeks illness. Necropsy showed enlargement of the liver, congestion of the kidneys, and exudative pleural pneumonia. *S. montevideo* (VI, VII: g,m,s—.) was isolated from its ovaries, spleen and lungs.

Iguana.—On Feb. 16, 1945, a male iguana (*Iguana tuberculata*) died at the same zoo. Necropsy showed an old head injury emaciation, flabbiness of the heart, delayed coagulation of the blood, congestion of the liver with several irregular areas of focal necrosis, ulceration of the upper intestine, and lung abscesses. From the liver, spleen, heart blood, bladder, and intestine, *S. manhattan* (VI, VIII:d:1,5.)) was isolated.

The cultures isolated from all of these animals were culturally and biochemically typical of Salmonella. The antigenic analyses were made by the writers, according to the Kauffmann-White Schema described by Edwards and Bruner[3], with the aid of a Salmonella Typing Kit furnished by the U. S. Army Medical School. The results have since been confirmed by Dr. P. R. Edwards, National Salmonella Typing Center, Lexington, Ky.

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DISCUSSION

To the best of our knowledge, this is the first published report on the positive identification of Salmonella from the order, Chelonia, (terrapins, turtles, tortoises). The autopsy records of the San Diego Zoo indicate, however, that an epizoötic which occurred in the same exhibit in 1935 may have been due to salmonellosis. None of the cultures isolated at that time was indentified by serological methods, and the available reports on the biochemical studies are not complete enough to warrant a positive diagnosis. The present reported isolations were made in March, 1944, and January and February, 1945.

S. sandiego was originally isolated during an outbreak of food poisoning at the San Luis Rey Mission near San Diego, California (Kauffmann[4]). The outbreak occurred in 1938, but this type has not been isolated since from man or animal in that area. As far as could be determined, there was no relation between the culture reported herein to the San Luis Rey Mission outbreak. *S. newport*, the second type isolated from the turtles, is one of the important causes of gastroenteritis in man and has also been isolated from a number of animals (Edwards and Bruner[5]). It is not a commonly isolated type in the San Diego area.

Caldwell and Ryerson[6] were the first to call attention to lizards being possible carriers of Salmonella types, in a report on the isolation of an organism which Kauffmann[7] later studied and called *Salmonella arizona*. This organism was isolated from sick gila monsters and was found to be pathogenic for horned lizards and chuckawallas, as well as for guinea pigs and rabbits. *S. arizona* has, however, since been placed by Edwards, Cherry, and Bruner[8] into the paracolon group because of its biochemical and antigenic reactions. The antigenic formula given by Edwards, Cherry, and Bruner is XXXIII,B,E: $z_4z_{23}z_{26}$, and it is designated as type 1 in their classification of the paracolons. It does have the "H" antigenic compounds, z_4z_{23}, in common with *Salmonella cerro*, which is true of many of the paracolons. The two Salmonella types, (*S. montevideo* and *S. manhattan*), reported herein, are believed to be the first true Salmonella to be reported from lizards.

One can only speculate on the method of transmission of infection to these animals, but it could have been food-borne from human carriers. The principal diet of the turtles consisted of lettuce, apples, carrots, melons, and spineless cacti and these food items could have been the source of infection, since Felsenfeld and Young[9] have shown that Salmonella may survive on stored vegetables for several weeks.

SUMMARY

Isolations of *Salmonella sandiego* and *Salmonella newport* from Galapagos turtles, *Salmonella montevideo* from a gila monster and *Salmonella manhattan* from an iguana are reported. It is believed that this is the first report on the isolation of true Salmonella from the reptilian order, Chelonia (turtles) and from the suborder of Squamata, which includes the lizards.

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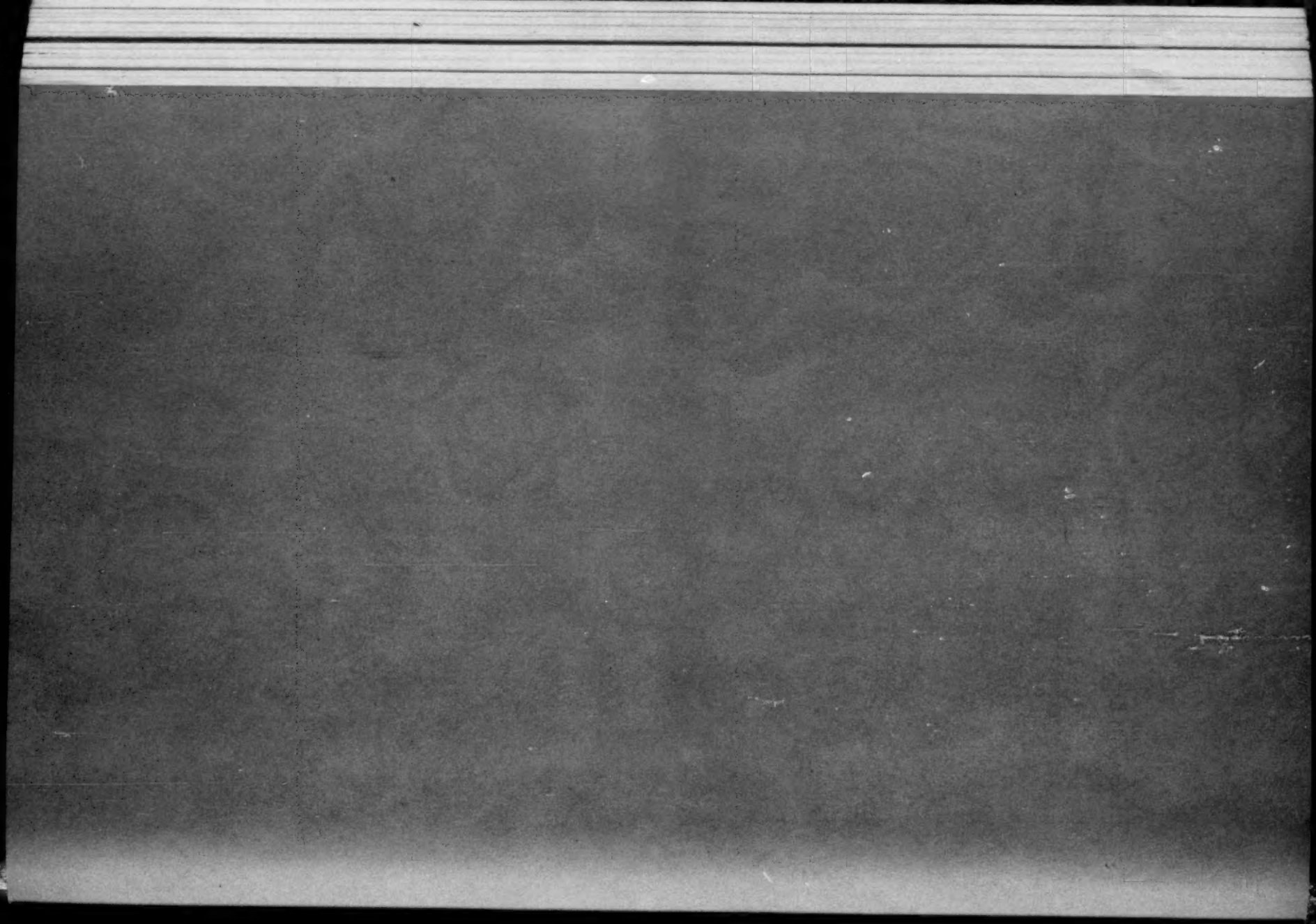
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